VIRAL INFECTIONS AS PREDISPOSING FACTORS FOR BACTERIAL MENINGITIS

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I dedicate this work to my brother, Akram and my parents

Declaration

I declare that this thesis has been composed by myself and that the research presented therein has been conducted by myself or under my direct supervision.

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Abstract

Epidemiological data suggest that viral infections might be predisposing factors for bacterial meningitis or carriage of type b *Haemophilus influenzae* (Hib), *Neisseria meningitidis* or *Streptococcus pneumoniae*, the three pathogens most commonly associated with bacterial meningitis. Non-secretors of ABO blood group antigens are over-represented among patients with bacterial meningitis and in populations affected by some outbreaks. The first objective of the study was to examine the hypothesis that non-secretors were also over-represented among patients with respiratory viral infections. Compared with the local population, there was a significantly higher proportion of secretors among patients with disease due to respiratory syncytial virus (RSV), influenza A virus, rhinovirus and ECHO virus. The hypothesis that Le^b and/or H-type 1 antigens present on cells or in body fluids of secretors might be receptor(s) for RSV was examined. Affinity purified molecules with Le^b or H type 1 determinants or synthetic receptor analogues did not decrease the infectivity of RSV for HEp-2 cells (a human epithelial cell line).

Bacterial attachment to mucosal surface is an important prerequisite for infection. The second major objective was to assess the effect of RSV-infection of HEp-2 cells on binding of bacteria responsible for meningitis. Binding of bacteria labelled with fluorescein isothiocyanate (FITC) to HEp-2 cells and RSV-infected HEp-2 cells was compared by flow cytometry. Strains of meningococci (3) and Hib (5) expressing antigens of different serogroups, serotypes and subtypes and a strain of Staphylococcus *aureus* bound significantly more effectively to virus infected cells compared with uninfected cells. Similar patterns of increased binding of unlabelled meningococci to monolayers of RSV-infected cells were also observed.

Studies to identify the changes on the cell surface associated with RSV-infection responsible for enhanced binding were carried out with one strain of meningococcus. Viral infection of HEp-2 cells did not enhance the expression of $Lewis^a$ antigen, a proposed receptor for bacteria in non-secretors. Monoclonal antibodies to the

attachment glycoprotein G of RSV decreased the bacterial binding to infected HEp-2 cells; but monoclonal antibodies to the fusion glycoproteins F did not affect the binding. Bacteria bound to infected cells also decreased the binding of monoclonal anti-G but not anti-F. Virus particles attached to bacteria. Monoclonal anti-G interfered with the viral attachment to bacteria but anti-F did not. These observations indicate that the G glycoprotein might act as a bacterial receptor.

G glycoprotein is a heavily O-glycosylated molecule. Deletion of O-linked sugars or removal of sialic acid from the surface antigens of uninfected or RSV-infected cells resulted in increased binding of bacteria. These data suggest that the protein moieties of the G glycoprotein and other molecule(s) native to HEP-2 cells might be the receptors for bacteria.

The present findings indicate that infection with RSV might be one of the factors contributing to increased colonization by the bacteria responsible for meningitis. Identification of the components on the infected HEp-2 cells involved in bacterial binding might be helpful in the search for bacterial epitopes associated with colonization by meningococci.

Abbreviations

BB	Blocking buffer
BBA	Boiled blood agar
BEC	Buccal epithelial cells
BI	Binding index
BSA	Bovine serum albumin
CNS	Central nervous system
CB	Coating buffer
CPE	Cytopathic effects
CSF	Cerebrospinal fluid
DPBS	Dulbecco's phosphate-buffered saline, solution 'A'
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyate
GC	Gonococcal culture
GM	Growth medium
HAI	Haemagglutination inhibition
Hib	*Haemophilus influenzae* type b
HSA	Human serum albumin
IFN	Interferon
Le	Lewis
MM	Maintenance medium
MNYC	Modified New York City medium
M.O.I.	Multiplicity of infection
NS	Non-secretor
OD	Optical density
PBS	Phosphate-buffered saline

p.f.u.	Plaque forming units
RSV	Respiratory syncytial virus
S	Secretor
TNF	Tumour necrosis factor
UEAI	*Ulex europaeus* lectin I

Chapter 1

General Introduction

Acute bacterial meningitis is a medical emergency that involves the leptomeninges and the subarachnoid space in a process of acute pyogenic inflammation. Early recognition of the condition, rapid detection of the etiologic agents, initiation of appropriate antimicrobial therapy and treatment of associated abnormalities and conditions are important for successful management. As the incidence of fatalities and complications is very high, identification of predisposing factors and the use of chemoprophylaxis and vaccination of those at risk are important.

1.1. Major pathogens

Haemophilus influenzae, Neisseria meningitidis and *Streptococcus pneumoniae* are the most common bacterial species causing meningitis. Greater than 90% of the endemic disease in children in the Sudan during 1985-1986 was caused by one of these three bacteria [Salih, 1990]. The death rate for the disease is high. That for meningitis due to *H. influenzae* varies from less than 5% in the USA to above 30% in Papua New Guinea [reviewed by Munson *et al.*, 1989]. Post-infection neurological and intellectual complications occur in 36% of patients with meningitis due to *H. influenzae* [Feigin, *et al.*, 1976]. The death rate is 10-20% in patients with disease due to *N. meningitidis* in industrialized countries and greater in developing countries [reviewed by Peltola, 1983]. The death rate approaches 80% in patients older than 50 years suffering from meningitis due to *Strep. pneumoniae* [Mufson *et al.*, 1982].

1.1.1. *H. influenzae*

H. influenzae is a small (0.1 X 0.3 um), non-motile, non-spore-forming,

Gram-negative coccobacillus. Its virulence is related to production of a polysaccharide capsule. The bacteria can be classified into 6 types, designated a-f, based on antigenic differences in the capsules. Type b *H. influenzae* (Hib) accounts for more than 95% of the invasive disease in children [reviewed by Moxon, 1990].

1.1.2. *N. meningitidis*

N. meningitidis (meningococcus) is a Gram-negative capsulate diplococcus with an average size of 0.6 X 0.8 um and flattened shape. The bacteria can be classified by seroagglutination based on the immunological specificities of the capsular polysaccharides into 13 serogroups: A, B, C, D, X, Y, Z, E, W135, H, I, K and L [reviewed by Appicella, 1990]. Nine of these serogroups are known to cause disease in humans, and most disease is caused by serogroups A, B or C [reviewed by Schwartz *et al.*, 1989]. The bacteria are also classified into serotypes on the basis of outer membrane proteins and 11 immunotypes of lipooligosaccharides present in the bacterial cell envelope [Zollinger and Mandrell, 1977]. Significant numbers of isolates are, however, non-typable. The endemic form of the disease appears to be caused by a heterogeneous collection of serotypes whilst a single serotype is usually involved in epidemics [Appicella, 1990].

1.1.3. *Strep. pneumoniae*

Strep. pneumoniae (pneumococcus) is a Gram-positive capsulate bacterium. Of the 84 recognized capsular types, 1, 3, 4, 7, 8, 9, 12 and 14 are most often associated with bacteraemia, including cases of meningitis [reviewed by Mufson, 1990].

1.2. Epidemiology

Hib affects mainly infants and children under the age of 6, mostly between 0-2 years;

and it is responsible for over 60% of cases of pyogenic meningitis in industrialized and developing countries [Greenwood, 1984; Schwartz *et al.*, 1989; Takala *et al.*, 1989; Nazareth *et al.*, 1992]. Endemic meningococcal meningitis is found mainly in children between 6 months and 5 years. The epidemic form of the disease is prevalent in a relatively older age group: children over the age of 10 and young adults [Geiseler *et al.*, 1980; Peltola, 1983; Jones and Kaczmarski, 1991]. Carrier-to-case ratio is higher in the endemic than the epidemic form of meningococcal disease [Greenwood, 1984]. Meningococcal disease also affects recruits in military institutions [Varela and Gilmore, 1971; Makela *et al.*, 1975; Griffiss and Bertram, 1977]. *Strep. pneumoniae* is most frequently associated with the disease in adults and is isolated from only 10% of children with meningitis [Greenwood, 1984]. All three pathogens cause the endemic form of the disease. Meningococci groups A and C, and less frequently group B, are associated with epidemics [Greenwood, 1984]. Most cases of meningococcal meningitis reported in the USA and Belgium occur in winter and early spring [reviewed by Peltola, 1983]. Peak incidence of the disease in England and Wales is in January [Jones and Kaczmarski, 1991]. Peak incidence of invasive disease, including meningitis, due to Hib is from October to January in England and Wales [Nazareth *et al.*, 1992].

The carriage rates vary with age, socioeconomic conditions and with the presence of ongoing epidemics. Meningococci are carried by 5-30% of individuals in non-epidemic periods, whilst the bacteria can be isolated from up to 100% of the population during epidemics [reviewed by Devoe, 1982]. Meningococcal carriage rate was determined in two groups of family contacts of meningitis patients. The first group was comprised of contacts from 21 families in which there was a case of meningococcal meningitis. Individuals in the second group were from 24 families in which there was a case of meningitis due to *H. influenzae* or *Strep. pneumoniae.* In

each group, 64 individuals were cultured within 48 hours of the diagnosis of the index case. Meningococcal carriage rate was 39% in the first group of family members compared to 1.6% in the second group [Olcen *et al.*, 1981].

Specimens from 41 households with one or more children, representing a cross-section of the social strata and in which there was no meningococcal disease were cultured bimonthly over a period of 32 months. Among 238 individuals tested, 18% were carriers of meningococci. Serogroup B was obtained from 50% of the positive cultures [Greenfield *et al.*, 1971]. Group specific carriage rates are, however, of little value in determining the population dynamics of meningococcal infection. In contrast, serotype specific carrier rate is a reliable indicator for the disease in a community [Griffiss *et al.*, 1977]. In another study the carriage rate among close contacts of patients was significantly higher than in the control population. Meningococci were isolated from 17% of 315 household contacts compared with only 2% in a comparable population of non-contacts [Cartwright *et al.*, 1991b].

Carriage rates have been estimated to be 3-5% for Hib and 50-80% for non-typable strains of *H. influenzae* [Moxon, 1986]. Turk [1984] has emphasized that the low carriage rate of Hib in children is due to a brief period of colonization (2 months) in children under 5; but, Hib was isolated from a much higher proportion of children (38.5%) when throat swabs from these children were cultured on multiple occasions during the first 5 years of life. The likelihood of occurence of secondary cases of meningitis due to Hib in the households within 30 days of occurence of the index case was 585 times higher than in the general population [Ward *et al.*, 1979]. OMP typing has confirmed that the secondary cases are caused by the same strain responsible for the respective index case [Kaplan *et al.*, 1983].

Pneumococci have been isolated from 5 to 70% of normal adults [reviewed by Mufson, 1990]. In a study over a period of 80 weeks, among 52 normal families (265

persons) residing in a low income housing project, 39% members acquired pneumococci in their throat; 85% of these subjects were under the age of 20 years [Suhs and Feldman, 1965]. Pneumococci and meningococci usually require several weeks to spread in a family following the initial nasopharyngeal acquisition by one of the family members [Suhs and Feldman, 1965; Greenfield *et al.*, 1971].

1.3. Pathogenesis of bacterial meningitis

1.3.1. Colonization

Colonization of the human nasopharynx might result in the bacteria remaining as part of the commensal flora on the mucosal surface, or they might spread to adjacent anatomical sites such as the middle ear or result in systemic infection [Beachey, 1981; DeVoe, 1982; Kaplan *et al.*, 1983]. Fresh isolates of *N. meningitidis* from the nasopharynx of carriers and patients are invariably pilate [reviewed by Heckels, 1989]. Pilate strains attach more effectively to human epithelial cells than isogenic bacteria from which pili are removed or non-pilate laboratory strains [Stephens and McGee, 1981].

There are conflicting reports on the importance of pili for attachment of *H. influenzae* to cells. All the clinical isolates of non-typable *H. influenzae* were found to be pilate, although the percentage of pilate bacteria in each isolate varied from 10-100% [Bakaletz *et al.*, 1988b]. In comparison, only 16% of the samples obtained from children with systemic Hib disease were found to be pilate [Mason *et al.*, 1985]. These data suggest that either pili are not necessary for nasopharyngeal colonization in children or loss of pili is associated with invasion of the host.

Pili of *H. influenzae* are not essential for binding of the bacteria to cells of different origins. Non-pilate bacteria attached more efficiently to HEp-2 cells than pilate

strains [Sable *et al.*, 1985]. In contrast, human buccal epithelial cells bound pilate strains more effectively [Pichichero *et al.*, 1982]. In addition to pili, ligands responsible for attachment to human nasopharyngeal cells have been demonstrated on the surface of Hib [Farley *et al.*, 1990].

1.3.2. Mucosal invasion by bacteria

Stephens *et al.* [1983] studied mucosal invasion by the bacteria using nasopharyngeal tissue obtained from healthy patients undergoing surgery to remove adenoid tissue. The resected tissue maintained in tissue culture medium was exposed to pilate strains of *N. meningitidis*. The bacteria attached selectively to microvilli of non-ciliated columnar epithelial cells; but at the same time, they were found to decrease the activity of ciliated cells. The microvilli of the cells binding the bacteria became distorted, long, thin and irregular in distribution. Such changes were not observed on the neighbouring cells to which meningococci did not attach. The microvilli restructured themselves around the bacteria. Bacteria were endocytosed within 6-12 hours of inoculation. The strains used in these assays were pilate, but no comment was made about change in pili during the attachment and entry process. In another study, pili were involved only in the initial stages of binding whilst irreversible binding was thought to result from covalent linkage between the two surfaces [Rogers, 1979].

In other experiments [Read *et al.*, 1991], inferior turbinates from patients with a history of nonallergic nasal obstruction were resected and maintained in nutrient medium. The cultures were incubated with non-typable strains of *H. influenzae* for 24 hours. Ciliary depletion was observed on the ciliated cells while both ciliated and non-ciliated cells showed cytopathic effects such as cytoplasmic blebs and mitochondrial abnormalities. The bacteria were characteristically associated with damaged ciliated and non-ciliated mucosal cells. Similar associations with damaged

epithelial cells were also observed for 5 other clinical isolates of the bacteria. It was suggested that, in the individuals with impaired mucociliary clearance, the products from the entrapped bacteria are detrimental to host tissues and damage the mucosa, paving the way for bacterial attachment.

A study comparing the initial stages in the pathogenesis of *N. meningitidis* and *H. influenzae* infections was conducted by Stephens and Farley [1991] using nasopharyngeal tissues of adenoids resected from children. Compared with meningococci, *H. influenzae* attached in larger numbers to the mucus lining the epithelium. Infection with *H. influenzae* led to breakdown of tight intercellular junctions so that the organisms were found in the intercellular spaces. Meningococci, in contrast, were endocytosed by non-ciliated cells. Both the pathogens produced factors that were ciliostatic and cytotoxic for ciliated cells.

Bacteraemia occurs in many forms of bacterial disease. Concomitant with the bacteraemia, or at various times thereafter, the bacteria spread to different organs and systems in the body. How these bacteria cross the blood-brain barrier is not known. A role for surface polysaccharides in predilection of these bacteria for the meninges has been suggested [McCracken, 1976].

1.3.3. The role of bacterial components in pathogenesis

1.3.3.1. The Capsule

The role of the bacterial capsule in pathogenesis of bacterial disease was recognized as early as 1920s by Dr. O.T. Avery (Rockfeller Institute, USA) who described the significance of the capsule in pneumococcal disease. The presence of a capsule appears to be important in the pathogenesis of meningococcal infection as non-capsulate strains are usually non-pathogenic [Salit and Morton, 1981].

Non-capsulate strains of *H. influenzae* account for the majority of localized disease, e.g., otitis media. Capsulate bacteria, in contrast, are responsible for systemic disease, e.g., meningitis [reviewed by St. Geme and Falkow, 1990].

Studies performed on infant rats inoculated intranasally with Hib indicate that the bacteria can be isolated from the blood stream within minutes of inoculation. Concomitant isolation of Hib from regional and distant lymph nodes in rats with sterile blood cultures 24 hours after intranasal inoculation with the bacteria indicates a direct invasion of nasopharyngeal blood vessels as an initial determinant of systemic infection [Rubin and Moxon, 1983]. The presence of a characteristic capsule is important for the bacteria to survive and replicate in the blood stream. In a rat model, genetically related transformants which expressed different capsular polysaccharides survived significantly less efficiently in the blood compared with capsulate strains of Hib [Zwahlen *et al.*, 1983].

Specific antibody levels might also be important in the pathogenesis of disease caused by Hib. Robbins *et al.* [1973] measured anti-capsular antibodies in 30 patients with epiglottitis, 30 patients with meningitis and 44 controls. The meningitis patients had markedly lower levels of anti-capsular antibodies compared with epiglottitis patients and controls. Similar trends in the antibody level in the patients with epiglottitis and meningitis due to Hib were found by Kayhty *et al.* [1981].

Masson *et al.* [1982] examined the effect of the group B meningococcal capsule on bacterial virulence for mice by comparing a wild type strain and a spontaneous isogenic mutant deficient in polysaccharide production. The loss of polysaccharides was found to be associated with dramatic loss of virulence.

1.3.3.2. Lipopolysaccharide/Lipooligosaccharide

The deleterious effect of endotoxins released by the bacteria on the host system can

be due to direct action on the tissues [reviewed by Jacobs and Tabor, 1990], disturbance caused in the central nervous system or release of cytokines such as tumour necrosis factor (TNF). Ducker and Simmons [1968] found that endotoxins introduced into the ventricles of the brain of dogs produced a massive oedema of lungs and haemorrhages in the subendocardium, viscera and adrenal glands. These changes were not associated with abnormalities, such as hypoglycemia, alterations in serum enzyme levels and plasma volume characteristic of endotoxic shock or with abnormalities caused by increased intracranial pressure. The quantities of the endotoxins administered intracranially were not sufficient to produce symptoms when administered intravenously. Systemically, bacterial endotoxins elicit the production of host factors such as TNF and cachectin that might in turn lead to shock and death [Beutler and Cerami, 1987]. Waage *et al.* [1987] measured the level of TNF in patients with meningococcal meningitis. The level was high in 10 of 11 patients who died compared with 8 of 68 survivors from the disease.

1.3.3.3. Pili

Pili are not associated with virulence of *Neisseria gonorrhoeae* in guinea pigs. Virulence of the bacteria was determined by their survival after the bacteria contained in polypropylene chambers were implanted in flanks of the animals. Loss of pili due to glucose-limited growth did not result in loss of virulence. In contrast, cystine-limited gonococci were pilate but avirulent [Keevil *et al.*, 1986]. Pili were, however, demonstrated on the bacteria isolated from CSF from a patient with meningococcal meningitis indicating a possible role for pili in the later stages of disease [Stephens *et al.*, 1982]. In contrast, pili were not demonstrated on the isolates of Hib from blood or CSF of infected rats [Kaplan *et al.*, 1983].

1.4. Factors affecting host susceptibility to bacterial meningitis

1.4.1. Maturation of infant immune system and correlation with the peak of disease

Meningococcal disease mainly affects children in the age range of 6 months to 5 years. Infants under 6 months are relatively protected by bactericidal antibodies acquired from the mother before birth. Infants born to mothers who are deficient in protective antibody or whose isotype-response is limited to IgM are not protected from the disease during this period. Appearance of specific antibodies against meningococci in children of 5 years and older is correlated with low incidence of disease. The adult levels of antibodies are reached by about 10 years of age [Goldschneider *et al.*, 1969]. Progressive reduction in the incidence of the disease after 2 years of age correlates with the rise of serum bactericidal antibodies against serotype antigens of the bacteria [Frasch, 1977]. Antibodies to polyribotol phosphate (PRP), the capsular polysccharide of Hib, are bactericidal. Minimal levels of anti-PRP antibodies are found in infants between 3 to 6 months of age [Moxon, 1990] which corresponds with high incidence of meningitis due to Hib in this age group.

1.4.2. Protective antibody response

Antibodies against the PRP capsular antigen of *H. influenzae* activate complement by both the classical and the alternative pathway and are opsonic [Steele *et al.*, 1984]. Robbins *et al.* [1973] found PRP to be present in a variety of Gram-positive bacteria. Bactericidal activity against *H. influenzae* was reduced by half by prior interaction of sera with bacteria containing PRP. Many human commensal *E. coli* strains also express epitopes that cross-react with *H. influenzae*. Anderson *et al.* [1972] demonstrated antigenicity of bacterial components of Hib other than PRP in 60% of 114 individuals examined. The sera from these subjects lacked detectable serum anti-PRP activity but still possessed bactericidal activity against Hib. Hib differed in their susceptibility to anti-somatic antibodies but were uniformly sensitive to

anti-capsular antibodies. Bactericidal activity of antibodies to non-capsular bacterial components was also demonstrated by Steele et al [1984].

Presence of G2m(n) allotype, a genetic marker of IgG2 antibodies, has been associated with high levels of the IgG2 isotype to PRP. The finding that the antibodies produced against bacterial antigens other than PRP can be bactericidal to Hib is further supported by the reports that individuals who are of G2m(n) n-/n- (not able to produce IgG2 isotype) are not more susceptible to these infections than G2m(n)-positive individuals [Takala *et al.*, 1991].

The antibody response during infection depends upon the maturity of the immune system. Kayhty *et al.* [1981] found that 61% of 77 patients >17 months of age had a >4 fold rise in the antibody level; in contrast, only 10% of 48 patients in the younger age group showed a similar rise.

Antibodies of all isotypes are produced against *N. meningitidis*, but they are not necessarily protective. Griffiss [1975] and Griffiss and Bertram [1977] demonstrated that the acute phase sera from 24 of 28 cases of meningococcal disease were inactive against the strains prevalent in the USA at that time; but, these became lytic when IgA was removed by immunoadsorption. Presence or absence of IgG in the sera was largely insignificant while IgM was bactericidal. The blocking effect of the IgA for serogroups B, Y and C was also observed at 12, 27 and 33 days after the first isolation of the bacteria.

Edwards *et al.* [1977] measured complement fixing antibodies against meningococci. They found an overall lag of 9 days between the acquisition of the bacteria and the appearance of significant amounts of antibodies. Peak titres were detected in the third week after the first positive culture of the bacteria.

The antibody response to group B polysaccharide is not as effective as to antigens of

the other serogroups. With monoclonal antibodies specific for the group B meningococcal capsule, it has been shown that cross-reactivity exists between the bacterial capsule and central nervous system (CNS), cardiac, hepatic and renal glycoproteins of the infant rat. As the animal matures, the cross-reactivity persists only in the CNS [Finne *et al.*, 1987]. Identification of α-(2-8) linked oligomers of sialic acid in polysaccharides of group B meningococci and in gangliosides of human brain tissue also suggested cross-reactivity [reviewed by Jennings *et al.*, 1985]. Poor immunogenicity of group B polysaccharide seems to be due to cross-reactivity with host tissues.

1.4.3. Role of complement

Complement factors are involved in the elimination of bacteria through their bactericidal activity and complement-mediated phagocytosis. Some complement deficiencies (C 5-9) are found to be associated with meningococcal disease [Fijen *et al.*, 1989].

In a rat model, the presence of complement factors is important in clearance of capsulate and non-capsulate strains of *H. influenzae* from the blood [Zwahlen *et al.*, 1983]. Bactericidal activity can be demonstrated in human sera in which complement factor C2 (classical pathway) or factor D and properdin (alternative pathway) were selectively inactivated [Steele *et al.*, 1984]. The evidence that Hib can activate the alternative pathway is also demonstrated by consumption of C3 in the absence of C4 in guinea pig serum incubated with the bacteria. PRP is not responsible for the activation since non-capsulate organisms can also activate this pathway [Quinn *et al.*, 1977]. It has, however, been suggested that the major contribution of complement in protection against Hib relates to its opsonic rather than its bactericidal activity [Moxon and Winkelstein, 1988].

1.4.4. Role of properdin

Properdin, a serum protein important in the alternative pathway of complement activation, contributes to protection against meningococcal infection. Densen *et al.* [1987] reviewed the literature reporting the occurrence of bacterial meningitis in individuals deficient in properdin. Meningococcal meningitis affected 8 of the 16 individuals with the deficiency and there was a high fatality rate in this group. In another study, 46 patients of 10 years or more with meningococcal disease due to rare serogroups X, Y, Z, W135 or 29E were investigated. Properdin deficiency was found in 9, C3 deficiency in 5 and deficiency of terminal components of complement in 9 [Fijen *et al.*, 1989]. This suggests that the alternative pathway is important in protection against the rare serogroups of meningococci. The hypothesis that complement provides the only bactericidal mechanism in the serum against these serogroups is unlikely since the rare serogroups are efficiently immunogenic [Griffiss *et al.*, 1981].

1.4.5. Nutritional status

The role of nutritional factors in susceptibility to meningitis has not been evaluated. These factors have, however, been examined for other infectious diseases. An association is found between chronic alcoholism and pneumonia caused by *H. influenzae*. In a retrospective survey of cases of pneumonia recorded between 1942 and 1971, 30% of the adults were alcoholics. A similar survey of the cases reported between 1970 to 1974 indicated alcoholism in 50 % of the adult patients [Levin *et al.*, 1977].

Klebsiella pneumoniae bind in larger numbers to nasopharyngeal epithelial cells obtained from children with mild to severe vitamin A deficiency than to the cells from a control population [Chandra, 1988]. The bacteria also attach more effectively

to the tracheal and buccal epithelial cells obtained from children suffering from protein-energy malnutrition compared with the control [Chandra and Gupta, 1990]. These studies were not controlled for presence of viral infections.

1.4.6. Trauma

Bacteraemia due to *H. influenzae* does not always lead to localized disease [Marshall *et al.*, 1979]. Trauma can convert unnoticed bacteraemia into an overt disease. Turk [1984] has reported the occurrence of Hib disease localized to traumatized parts of the body including meningitis in patients with recent history of head injury without fracture.

1.4.7. Socio-environmental conditions

In a case-control study of 108 patients with meningococcal disease and 103 controls, factors such as bacterial carriage and acute respiratory infections were significantly associated with disease. Crowding was not directly associated with the disease, although it influenced the incidence of the disease by increasing the carriage rate and the incidence of acute respiratory infections [Moore *et al.*, 1990]. In contrast, crowding was not associated with the carriage rate in a study conducted by Stuart *et al.* [1988]. They found associations, which were not statistically significant, between low socio-economic conditions and the disease in 83 cases of the disease compared with 88 controls. The houses of the patients were generally more damp and less heated than the average.

Carriage is associated with active smoking. In a study carried out by Blackwell *et al.* [1990], 46% of 37 smokers were carriers compared with 25% carriers in 349 non-smokers. Passive exposure to cigarette smoke did not predispose to carriage. In contrast, passive smoking was a risk factor for disease in adults over 20 years (n=10)

and in children under 12 years (n=40) compared with similar numbers of controls. [Stuart *et al.*, 1988]. Smoking, both active and passive, was associated with carriage of meningococci in a study of Greek military recruits; and there was a strong correlation between carriage and number of cigarettes smoked per day [Blackwell *et al.*, 1992]. A similar pattern of association was observed between carriage and smoking in 87 pairs of carriers and non-carriers of 12 years or older [Stuart *et al.*, 1989]. The risk of carriage in active smokers was proportional to the number of cigarettes per day. No other factor such as nasal symptoms, tonsillectomy or adenoidectomy was associated with carriage in this study. Tonsillectomy, however, was found to be associated with carriage in another study [Kristainsen and Elverland, 1984]. Fifteen of the 62 individuals after tonsillectomy compared with none of 62 controls converted from non-carrier to carrier state. The authors did not, however, comment on the higher incidence of carriage in the subjects before tonsillectomy compared with the controls.

Gonococcal infection in military recruits is suggested to contribute to susceptibility to meningococcal disease. Lipooligosaccharides of the two species are antigenically cross-reactive. The amount of bactericidal activity against meningococci of serogroups A, B and C is negligible among patients with gonorrhoea. This suggests that gonococci might be adsorbing serum antibodies bactericidal to meningococci and thus enhancing the susceptibility to infection by the latter [Winstanley *et al.*, 1983].

1.4.8. Secretor status and susceptibility to infectious disease

1.4.8.1. Genetics and expression of ABH and Lewis blood group antigens

The ability to secrete the water-soluble form of the ABH blood group antigens in body fluids is determined by the secretor (S) gene located on chromosome 19 [Watkins, 1980]. The secretor gene is in the same linkage group as the Lewis gene

and both code for fucosyl transferases that modify the type 1 precursor chain. The expression of H and Lewis antigens on cells and in body fluids of secretors and non-secretors is summarized in Table 1.1. Non-secretors express only Lewis^a while secretors express Lewis^b and variable amounts of Lewis^a.

A, B, H and Lewis blood group substances are found in two forms, glycolipid mainly present in the cell membrane and glycoprotein mainly present in the secretions. The glycoproteins are macro-molecules composed of 10-20% amino acids and the remainder is carbohydrates. The carbohydrate moieties consist of a basic precursor chain to which different monosaccharides are added by cellular enzymes. Of the 4 types of precursor chains (Table 1.2), type 1 and type 2 appear to be the most common. Type 1 chains are the main carriers of blood group determinants in secretions and on the lining and glandular epithelia. In comparison, blood group antigens on theerythrocytes are mainly type 2 precursor chains [Tilley *et al.*, 1975; Oriol *et al.*, 1986]. The interactions of the Se gene- and Le gene-coded enzymes on the type 1 precursor chain are illustrated in Fig 1.1.

Secretor status is usually determined by a haemagglutination inhibition assay with saliva [Mollison, 1979]. Determination of Lewis blood group by agglutination of red cells or detection of these antigens in saliva has also been used to determine secretor status [reviewed by Mourant *et al.*, 1976].

1.4.8.2. Secretor status and infectious diseases

Non-secretors are significantly over-represented among patients with a variety of bacterial diseases and superficial yeast infections. Non-secretion is also associated with some autoimmune diseases for which infectious triggers have been proposed (Table 1.3).

Non-secretion of blood group antigens is associated with invasive disease due to

Table 1.1. Distribution of H and Lewis antigens in the secretions and on cells of secretors (S) and non-secretors (NS).

Antigen	NS		Se	
	Secretions	cells	Secretions	cells
Le^a	+	+	+	+
Le^b	-	-	+	+
H-type 1	-	-	+	+
H-type 2	-	+	-	+

Table 1.2. Four type of precursor chains of blood group antigens.

Type 1	Galβ1 → 3GlcNAcβ1 → R
Type 2	Galβ1 → 4GlcNAcβ1 → R
Type 3	Galβ1 → 3GluNAcα1 → R
Type 4	Galβ1 → 3GluNAcβ1 → R

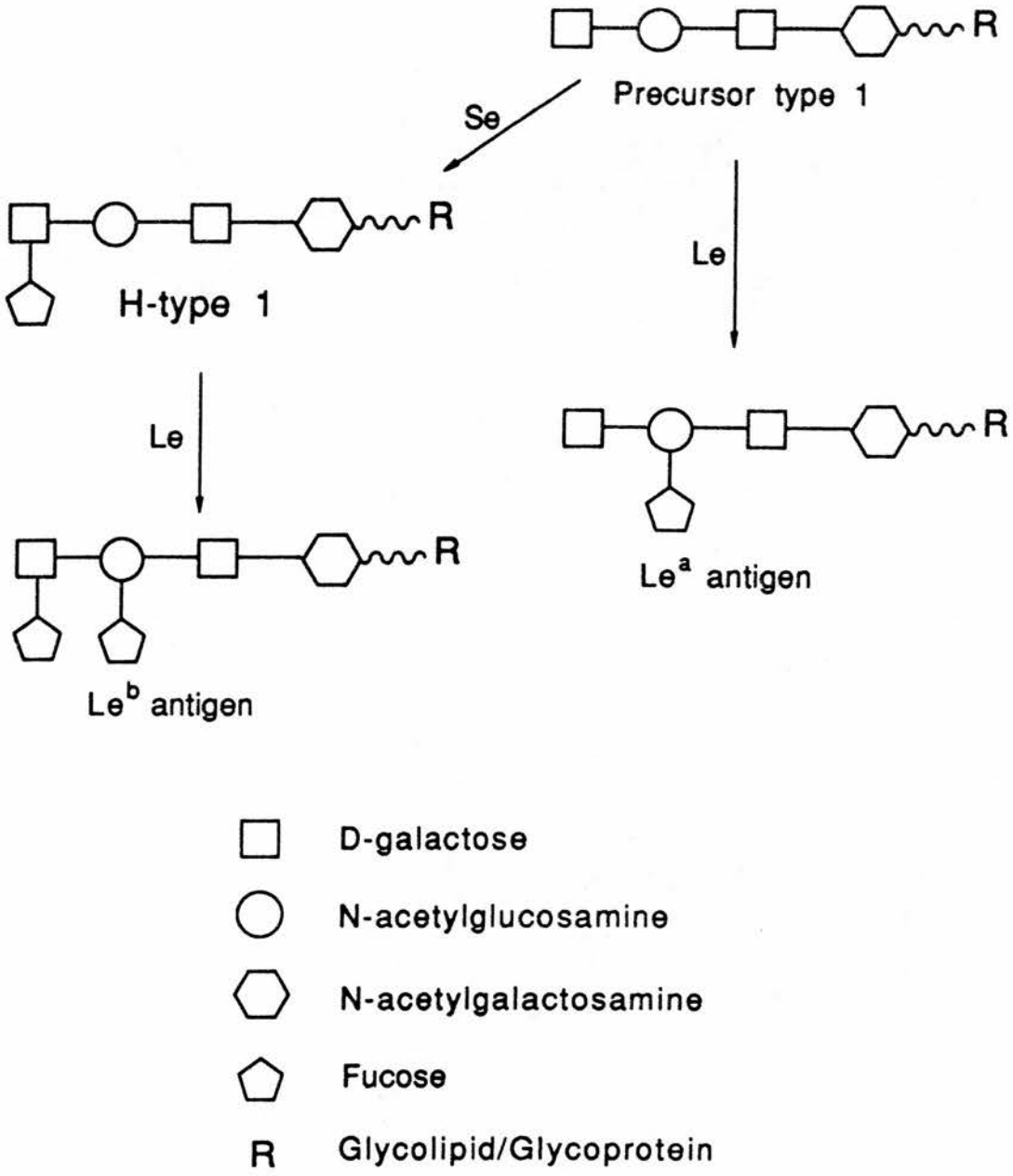


Figure 1.1. Modification of type 1 precursor chain by fucosyltransferases coded by the Lewis and the secretor genes.

Table 1.3. Association between non-secretors and susceptibility to diseases.

Infectious agent/disease	**Reference**
Respiratory tract	
Strept. pyogenes (Carrier and Rheumatic fever)	Haverkorn and Goslings, 1969; Glynn *et al.*, 1959
Neisseria meningitidis	Blackwell *et al.*, 1986a
Strept. pneumoniae	---"---
Haemophilus influenzae	Blackwell *et al.*, 1986b
Gastrointestinal tract	
Vibrio cholerae	Chaudhuri and Das-Adhikary, 1978
Peptic ulcers	reviewed by Mourant *et al.*, 1978
Urinary tract	
E. Coli	Kinane *et al.*, 1982 Blackwell *et al.*, 1987b
Oral cavity	
Candida albicans	Thom *et al.*, 1989
Dental caries	Holbrook and Blackwell, 1989
Autoimmune diseases	
Ankylosing spondylitis	Shinebaum *et al.*, 1987
Insulin dependent diabetes mellitus	Blackwell *et al.*, 1987a
Graves' disease	Collier *et al.*, 1988

meningococci, Hib and pneumococci, the 3 major bacterial pathogens causing meningitis. The secretor gene is inherited in a mendelian-dominated pattern, and 75-80% of European populations are secretors while 20-25% express the recessive non-secretor phenotype. Regional variations in these phenotypes are also reported [Lincoln and Dodd, 1972; Mourant *et al.*, 1976]. In areas where there have been prolonged outbreaks of meningococcal disease, Iceland, Stonehouse, Gloucestershire and Plymouth in the UK, there are significantly higher proportions of non-secretors in the population [Blackwell *et al.*, 1986(a); Eriksson *et al.*, 1986; Blackwell *et al.*, 1989(c); Blackwell and Weir, 1990].

Several hypotheses have been suggested to explain the increased susceptibility of non-secretors to bacterial diseases. 1) Lewis^a antigen present in higher amounts in non-secretors might be a receptor for microorganisms. There is some evidence that Lewis^a antigen might be a receptor for *Candida albicans* [May *et al.*, 1989; Tosh and Douglas, 1991; Aly, 1992]. Buccal epithelial cells (BEC) obtained from non-secretors bound staphylococci more effectively compared with BEC from secretors [Saadi *et al.*, submitted for publication]. 2) Non-secretors might be more susceptible to bacterial diseases because of lower level of serum and salivary antibodies. Recent studies have not confirmed early reports which suggested lower levels of serum or secretory IgA in non-secretors [Blackwell, 1989]. Non-secretors do, however, have significantly lower levels of salivary IgM [Zorgani *et al.* in press]. 3) The secretor gene and the gene for the third component of complement (C3) are in the same linkage group. C3 levels were found slightly lower in non-secretors compared with secretors; and 7 of 8 subjects whose C3 levels were below the lower limits of the normal range were non-secretors [Blackwell *et al.*, 1988].

1.4.9. Viral infections predisposing to bacterial diseases

Virus infections are associated with enhanced colonization and disease caused by a

variety of bacterial pathogens. Most studies have investigated the association between influenza virus and bacteria causing pneumonia, probably because of the high incidence of lung complications among the populations experiencing epidemics in New York during 1957-1958 [Louria *et al.*, 1959], in Connecticut during 1957 [Petersdorf *et al.* 1959] and in Hong Kong during 1969 [Schwarzmann *et al.*, 1971]. Increased adherence of staphylococci to the pharyngeal cells of volunteers infected with attenuated influenza virus was observed by Musher and Fainstein [1981]. This finding was repeated using MDCK cells (a canine kidney cell line) infected with the virus [Sanford *et al.*, 1986]. *Strep. pneumoniae* bound more readily to the tracheal epithelial cells of influenza virus infected mice compared with the controls [Plotkowski *et al.*, 1986].

Gram-negative bacilli are not normally present in the oropharyngeal flora. A survey was undertaken over a period of 11 months involving 89 staff house officers to assess the effect of clinical viral respiratory tract infections on the isolation of Gram-negative bacilli and staphylococci. The results showed that colonization by the bacteria increases significantly during episodes of viral illness compared with illness free periods [Ramirez-Ronda *et al.*, 1981].

1.4.9.1. Evidence for virus infections as predisposing factors to infection by bacteria causing meningitis

1.4.9.1.1. *N. meningitidis*

The elderly are not generally susceptible to meningococcal disease. In contrast, an outbreak of meningococcal meningitis in a geriatric ward affected 11 of 55 women, 27 of whom were suffering from A2 influenza virus infections. Significant association between the two pathogens was also shown by the serological evidence in these patients [Young *et al.*, 1972]. Data from a military training center covering a

period from 1967 to 1971 indicated a lag of 7-10 days between acute respiratory diseases necessitating hospitalization and meningococcal disease [Edwards *et al.*, 1977]. It is not clear from the study which pathogens were involved in the acute respiratory diseases. Upper respiratory tract infections with adenovirus, parainfluenza, respiratory syncytial virus (RSV) and rhinovirus or mycoplasma have been reported to be risk factors for epidemic disease due to group A *Neisseria meningitidis* [Moore *et al.*, 1990].

In a survey of patients with meningococcal disease, 46% of 69 cases had prodromal symptoms affecting the upper respiratory tract within 1 week before onset of signs and symptoms of the disease [Olcen *et al.*, 1979]. An investigation of household contacts of patients with meningococcal disease showed a bacterial carriage rate of 61% among the individuals with upper respiratory symptoms compared with only 14% among those with no symptoms [Olcen *et al.*, 1981].

Infections by adenovirus and influenza B virus were detected by isolation of the viruses or by serological methods in a study involving 160 cases of meningitis due to *H influenzae* and *N. meningitidis* and 138 controls. The viruses were significantly associated with the bacterial disease [Krasinski *et al.*, 1987]. The control group selected in this study was not ideal since they were not age-matched with the patients and were admitted to hospital for elective surgery, supposedly free of respiratory tract infections. The finding of an association of adenovirus infection with meningococcal disease has been confirmed [Moore *et al.*, 1990]. In a previous study, however, adenovirus infections or vaccination with the attenuated strain did not change the pattern of dissemination or invasiveness of meningococci in military recruits [Artenstein *et al.*, 1967].

A recent report [Cartwright *et al.*, 1991a] indicates that 38% of 53 meningitis patients of 10 or more years of age compared with 6% of individuals in a control group had

high titres of antibodies against influenza A virus. Possible associations with other respiratory tract viruses were not reported in this study. Analysis of the data in this study obtained from the questionaires supplied to the patients and the control group showed that a high proportion of meningitis patients suffered from a flu-like syndrome during the previous 2 months.

1.4.9.1.2. *H. influenzae*

Respiratory syncytial virus (RSV) infection of children has been significantly associated with bacterial superinfection. Bacteria were cultured from 47 of 194 RSV infected children. *H. influenzae* was the most common bacterium, isolated from 50% of the 47 children. [Meguro *et al.*, 1988]. *H. influenzae* and *Strep. pneumoniae* were isolated from 32% of 66 children suffering from RSV infections [Wahlgren *et al.*, 1984]. The bacteria were not typed in this study but the samples were obtained from blood or lungs indicating systemic infection caused by invasive strains. The data in both of these studies were not compared with any control group since the studies were primarily conducted for comparison of antibiotic treatments and X-ray findings. The finding is, however, significant when compared with the low carriage rate of 3-5% of Hib in a similar age group [Moxon, 1986].

A 14-year prospective study of 110 children indicated that attacks of RSV and adenovirus infections were significantly associated with colonization and disease (e.g., otitis media) caused by *H. influenzae* and *Strep. pneumoniae* [Henderson *et al.*, 1982]. Examination of sera from 51 children with viral respiratory tract infections for the concomitant presence of the bacterial antigens or specific antibodies to *H. influenzae* and pneumococci indicated a higher incidence of mixed viral and bacterial infections compared with earlier reports [Hietala *et al.*, 1989].

Children hospitalized during an epidemic of RSV infection and during the

post-epidemic period were screened for the presence of RSV and evidence of coinfection by bacteria. The incidence of infection by *H. influenzae* and *Strep. pneumoniae* based on a significant rise of the antibody titre or detection of pneumococcal antigens in serum or urine was significantly higher in 90 RSV-positve children during epidemic than 91 RSV-negative children admitted with similar complaints during the epidemic or with 99 children after the outbreak [Korppi *et al.*, 1989]. The proportion of RSV-positive cases and the incidence of co-infection by bacteria in the post-epidemic group is not provided in the data. Associations with other bacteria such as meningococci were not examined in the study. These data suggest that infection with a variety of respiratory tract viruses enhances susceptibility to bacterial diseases.

1.4.9.1.3. *Strep. pneumoniae*

Children (69) suffering from respiratory illness were compared with a control group of 28 age-matched surgical patients who were free of infections. Both viruses (mostly parainfluenza 3 and RSV) and bacteria (pneumococci and streptococci) were detected in 17% of patients compared with 4% in controls [Nickol and Cherry, 1967]. The bacteria were detected by throat swab cultures and the viruses were detected either by positive culture or by serological evidence. Simultaneous spread of rhinovirus and pneumococci occurred more commonly in families [Gwaltney *et al.*, 1975] and in apparently well, pre-school children in a study carried out in a village community [Taylor *et al.*, 1988].

1.4.9.2. Contribution of respiratory viral infections to bacterial diseases or carriage

Factors proposed to contribute to enhanced susceptibility to bacterial diseases associated with viral infections include: immunosuppression; enrichment of local growth conditions; enhanced binding to virus infected cells. Several common viral

infections cause some degree of immune suppression [reviewed by Jakab 1981(a, b); Mims, 1986; Babiuk *et al.*, 1988]. Influenza virus infection causes impairment in the function of alveolar macrophages [Jakab, 1982], reduction in chemotaxis of monocytes [Kleinerman *et al.* 1975] and reduction in lymphocyte responsiveness and proliferation of T-suppressor cells [Gardner, 1981]. Influenza viruses also interfere with the chemotactic and phagocytic function of polymorphonuclear leucocytes (PMN) [Craft *et al.*, 1976; Larson and Blades, 1976; Larson *et al.* 1980; Martin *et al.*, 1981]. Similar effects on the function of PMN were found with RSV [Craft *et al.*, 1976]. The resulting immunosuppression might reduce the response to unrelated bacterial antigens.

Certain bacteria chelate iron which is essential for their growth [Morse *et al.*, 1988; Chen *et al.*, 1989]. Infections of the respiratory tract caused by viruses can result in focal microhaemorrhages favouring growth of these bacteria. Viral infections disrupt the lining epithelium and cause local oedema and tissue injury. These changes predispose to bacterial invasion and jeopardize local immune functions. For instance, local anoxia caused by inflammation decreases the oxygen-dependent bactericidal activity of tissue macrophages while the exudate can enhance bacterial growth. Lack of ciliary function due to destruction of cilia caused by some viruses can result in impeded clearance of invading bacteria [Camner *et al.*, 1973]. Viral respiratory infection is proposed to enhance aerial dissemination of bacteria harboured by babies [Eichenwald *et al.*, 1961].

Binding to epithelial cells is an important step for most bacterial infections. It depends upon a number of factors including cellular surface chemistry and presence of specific receptors on host cells. Changes in these factors might alter the attachment of bacteria. For instance, pharyngeal cells obtained from children with malnutrition bound more bacteria compared with cells from healthy children [Chandra, 1988; Chandra and Gupta, 1990]. Similarly, alteration of cell surfaces

caused by decreased amounts of fibronectin in the respiratory mucosa in patients with cystic fibrosis was associated with binding of bacteria to the mucosal cells which normally do not bind those bacteria [Woods *et al.*, 1980]. Cell lysis caused by viruses increases the level of proteases in the lining secretions which, in parallel with the enzymes released by some bacteria, can degrade fibronectin on the surface of surrounding cells thus enhancing bacterial binding. The degrading effect of proteases on fibronectin during viral infections has been described [Keski-Oja *et al.*, 1987]. These workers have also reviewed the effect of infection of chicken fibroblasts with Rous sarcoma virus in decreasing biosynthesis of fibronectin. Decreased level of fibronectin on nasopharyngeal cells in hospitalized patients was associated with increased frequency of isolation of Gram-negative nasopharyngeal flora [Murphy and Florman, 1983].

Viral infection can affect the cells in many different ways. Invasion by viruses does not always lead to cellular lysis; selective impairment of metabolic, physiological or genetic functions can result from subtle, non-lethal damamge to the cells. Infection of human PMN with a recombinant influenza A virus stimulates intracellular phosphokinase and enhances phosphorylation of proteins. The process is, however, decreased on subsequent exposure of the cells to the virus [Caldwell *et al.*, 1988].

Human immunodeficiency virus (HIV) induces a glycosyltransferase in a human T cell line resulting in neo-expression of Lewis^y antigen on the infected cells. The antigen is detected on the peripheral T lymphocytes from AIDS patients and not on the lymphocytes from healthy individuals. The expression of the antigen on the cells is found to be of prognostic value [Adachi *et al.*, 1988]. Hepatitis B virus also causes neoexpression of Lewis^x molecules on the surface of Kupffer cells of the liver [Okada and Tsuji, 1990]. Infection of CV-1 and HeLa cells with Rous sarcoma virus results in enhanced production of a characteristic cellular protein involved in glucose transport across the cell membrane [Peluso *et al.*, 1978]. Hynes and Bye [1974]

described alterations to the cell surface proteins caused by viruses and association of these alterations with cell transformation. It has been reported that 90% of RSV infective particles remain associated with the membrane of infected cells [Levine and Hamilton, 1969], but fusion of the budding virus with the infected cell occurs very rarely [Bachi, 1988]. This suggests that RSV infection of the cells results in decreased expression of the receptors for viruses.

Glycoproteins of enveloped viruses have also been implicated in alterations of the surfaces of infected cells [Sanford *et al.*, 1978]. In infections by naked viruses such as Picornaviruses, the maturation and assembly of the virion takes place intracellularly and the viruses are released by cytolysis. In contrast, replication of enveloped viruses, e.g., (-) stranded RNA viruses, involves the synthesis of viral structural glycoproteins and their insertion into the plasma membrane. Nascent virions are transported to the surface where they mature by acquiring the glycoproteins and are extruded from the cell by a budding process. The viral glycoproteins make aggregates displacing the host structures on the membranes. By virtue of these biochemical changes, the viruses impart to the cells a new antigenic specificity which could be used for recognition by the immune system. These new antigens on the surface of cells might also provide receptor sites for other pathogens and might be responsible for enhanced binding of bacteria to virus-infected cells reported in some studies.

There are some enveloped viruses such as herpesviruses which assemble and mature at the inner lamella of the nuclear membrane and are transported to the surface of the cells through cisternae of cytoplasmic membranes and in the vesicles. These also cause similar changes on the surface of cells.

1.5. Experimental models for examination of bacterial binding

Many experimental models have been used to investigate virus induced alterations in

attachment and invasion by bacteria. Human volunteers are rarely used as models for studies of bacterial pathogens. In vitro assays on human cells and cultures of cells and organs from human sources are employed but there are some limitations.

1.5.1. Buccal and pharyngeal epithelial cells

Human buccal epithelial cells have been used in many studies of bacterial attachment [Craven *et al.*, 1980; Salit and Morton, 1981; Stephens, 1989]. Easy availability of the cells is offset by the fact that they are not uniform in size, degree of maturity or viability. They might already have bacteria attached to their surface from oral flora which can affect the results of binding assays. The cells are not the normal site of attachment of bacterial pathogens. Bacteria can attach to these cells but unlike *in vivo* target cells, they do not penetrate the membrane of these cells. Different samples of buccal epithelial cells can vary in surface chemistry due to molecules from the food taken just before sampling or to inherited differences between individuals, e.g., blood groups. Variations in binding of bacteria to cells from the same donor have been shown [Tramont and Wilson, 1977]. Use of buccal epithelial cells to study the effect of virus infection on bacterial binding is further hindered by the inability of these cells to be infected with respiratory viruses.

Pharyngeal cells can also be obtained relatively easily. Their use in *in vitro* bacterial attachment assays is limited by factors described above. Pharyngeal cells, however, are relevant to the study of the viral-bacterial association since virus-infected cells can be obtained from virus-infected hosts.

Fainstein *et al.* [1980] compared bacterial binding to pharyngeal cells obtained from 21 individuals who had nasopharyngeal symptoms but were culture negative for influenza virus with cells from 10 healthy subjects infected with an experimental

vaccine strain of influenza virus and with cells from 21 age-matched, symptom-free controls to study the effect of viruses on bacterial binding. Increased binding of *Staph. aureus* to the infected cells compared with uninfected cells was observed. In contrast, this pattern of binding was not observed for some other bacteria used in the assay. No comment was made about the bacterial species which might have been present in the subjects in different groups. Evidence for infection by viruses in the individuals with nasopharyngeal symptoms was not provided in the study.

1.5.2. Tissue/organ cultures

Nasopharyngeal tissues from adenoids [Stephens, 1989; Stephens and Farley 1991] and nasal tissues from inferior turbinates resected from patients with non-allergic symptoms [Read *et al.*, 1991] were obtained for the study of pathogenesis of *N. meningitidis* and *H. influenzae* infections. This model might accurately reflect the events happening in natural infections, but it has disadvantages. The tissues are difficult to obtain. The features related to genetic make up of the donor and the disease for which the donor was treated are reflected in the resected tissue. Patients undergoing surgery are presumably free of viral infections. So far, no attempt has been made to infect these organ cultures with viruses in vitro.

Organs, however, can be used from animals naturally or experimentally infected with viruses. Tracheas from mice experimentally infected with influenza virus A and killed on 2nd, 4th or 6th day post-infection were exposed to a suspension of radiolabelled *Strep. pneumoniae* for 90 minutes. Radioactivity from the tracheas of the infected mice was found to be significantly higher than from the tracheas from uninfected mice indicating that influenza virus enhances binding of streptococci. The bacteria were found to be attached to flat-surfaced epithelial cells which replaced ciliated columnar epithelial cells in the mucosa of the infected mice and to basement membrane on the denuded areas of the tracheas as shown by elecron microscopy

[Plotkowski *et al.*, 1986]. Electron microscopic findings of bacterial attachment to the mucosa of the tracheas from uninfected mice were not reported in the study. Organ cultures of tracheas from chinchillas infected with influenza A virus have been used to study the effect of virus infection on the tracheal histopathology and on binding of *H. influenzae*. Non-typable strains bound more effectively compared with Hib. Increased titres of Hib were recorded in the nasal washings from infant rats that had been inoculated with influenza A virus followed by bacteria 48 hours post-infection [Michaels and Myerowitz, 1983]. Binding of nontypable *H. influenzae* to chinchilla tracheal tissue infected with influenza A virus was decreased at 72 hr post-infection [Bakaletz *et al.*, 1988a].

1.5.3. Animal models

Animal models have also been used for the study of virus-bacterium association. A maximum delay in clearance of *Pasteurella pneumotropica* from the lungs of rats infected with Sendai virus after daily challenge with the aerosolized bacteria was observed during 6-9 days post-infection [Jakab, 1981a].

Use of organs dissected from animals and animal models is, however, not relevant for assessment of effect of viral infections on the bacteria causing meningitis since most common bacteria responsible for the disease (Hib, meningococci and pneumococci) are exclusive human pathogens. Animals, however, have been used to study isolated events in the pathogenesis of the disease.

1.5.4. Cell culture models

Cell culture models have been used for the study of cytotoxicity caused by bacteria, bacterial attachment, and the effect of viral infections on this attachment. Primary cells have been employed for these studies but are difficult to establish and maintain.

No significant difference was observed in the binding of *Staph. aureus* and other bacteria causing bovine respiratory diseases to cells from bovine embryonic lung infected with bovine parainfluenza virus [Toth and Gates, 1983]. A primary endothelial cell culture established from newborn umblical cord was used to examine the binding of *Staph. aureus* to human endothelium in the pathogenesis of bacterial endocarditis [Ogawa *et al.*, 1985]. Complications such as variations in the source of the tissues and, often, pathology of the organ from which the tissue in question is resected make the primary cell culture model less than ideal for studies of virus-bacterium interaction.

Permanent cell lines of tumour origin are easy to handle and can be maintained aseptically in standard conditions. Stephens [1989] reviewed the use of different models including HeLa cells and HEp-2 cell line (human epithelial cell lines) in studies of the binding of *Neisseria* species. Permanent cell lines were found suitable for the studies. Chang epithelial cells (derived from human conjunctiva) were used to determine the role of bacterial pili in the attachment of *H. influenzae* [St. Geme and Falkow, 1990]. HeLa and Detroit cells (a human epithelial cell line) were used to study the effect of cellular infection with measles virus and adenovirus on binding of bacteria [Selinger *et al.*, 1981]. Sanford *et al.* [1978] used MDCK cells to examine the effect of influenza virus infection of the cells on the attachment of streptococci. The bacteria attached to the infected cells only. The attachment could be blocked by pre-treatment of the infected cells with an antibody to the viral glycoproteins expressed on the surface of the infected cells indicating that the viral glycoproteins might be the receptors for the bacteria. Staphylococci did not attach to MDCK cells in this study; but, attachment was observed in a later study by the same group [1986]. The cells in this study were used to analyse the effect of influenza virus infection, the effect of enzymes and changes in other conditions in the assay on the attachment of staphylococci. The reason for the discrepancy between the two findings of the

attachment of staphylococci to MDCK cells was not discussed in their report.

1.5.5. Detection of bacterial binding

Methods commonly used for assessing bacterial binding to cell cultures include: light microscopy of stained monolayers of the cells [Selinger *et al.*, 1981; Musher and Fainstein, 1981]; fluorescence microscopy [Sanford *et al.*, 1978]; interference contrast microscopy [Andersson *et al.*, 1986]; electron microscopy [Childs and Gibbons, 1988; Stephens *et al.*, 1983]; radioassays [Ogawa *et al.*, 1985; Sanford *et al.*, 1986]; and pour plate method, counting colony forming units of bacteria in the supernatant after incubation with cell monolayers [Ogawa *et al.*, 1985].

Direct microscopy is simple, cheap and allows visualization of the process being studied. It is, however, not accurate for quantitation of large numbers of bacteria or for large experiments. Radioassays are also limited in value since the manipulation of bacteria for the uptake of the label can affect bacterial binding. Leakage of radioactive material from the labelled bacteria and background disturbance in detection of radioactivity in these assays also make the assessment more complicated. The use of electron microscopy is mainly limited to the study of mechanisms of bacterial attachment to the cells and bacterial pathogenicity. It cannot be used to demonstrate adherence with confidence since the results cannot be generalized due to patchy distribution of bacteria bound to cells and the area of tissue visualized by the micrscope is limited.

Flow cytometry has also recently been applied in our laboratory for study of binding of bacteria [Rahat, 1990]. The flow-cytometer can detect fluorescein-labelled bacteria on cells. The proportion of cells with bacteria attached and mean level of fluorescence can be obtained using this method. Although mean fluorescence recorded on cells is proportional to the numbers of bacteria attached, it cannot be

expressed in terms of numbers of bacteria because the level of fluorescence obtained with one bacterium on a cell is difficult to determine accurately. The method is, however, rapid, objective, precise and analyses a large number of cells in each sample.

1.6. Human respiratory syncytial virus (RSV)

Although it has been suggested that infection with influenza virus might be a factor predisposing to bacterial infections, RSV was chosen as the model for the studies presented here for the following reasons. RSV is an ubiquitous, important viral respiratory pathogen. The virus causes lower respiratory tract infections, bronchiolitis and pneumonia, mainly in infants, the elderly and immunocompromised hosts [Hall, 1980]. Reinfection is commonplace but the severity of the infection decreases with frequency of exposure and age. RSV causes upper respiratory illness and otitis media in adult populations and military personnel [Hers *et al.*, 1969; Sanford, 1975; Henderson *et al.*, 1979]. Epidemiological studies indicate that half of all children are infected in their first year of life and virtually all by the age of two [Henderson *et al.*, 1979]. Epidemics of RSV infections recorded in Washington DC between 1957 and 1970 occurred primarily in late autumn, winter and early spring each lasting for about 5 months [Kim *et al.*, 1973]. A peak incidence of RSV infection in winter and early spring was also recorded in England and Wales during 1980-1982 [Noah, 1989] and during 1989-1991 [Anonymous, 1991]. An MRC survey over two years also suggested a high incidence of RSV infections among children, hospitalized or in general practice, between October and March [Gardner, 1973]. This pattern fits the incidence of bacterial meningitis better than that observed for influenza virus infection [Noah, 1989]. Figure 1.2 summarizes the relative incidence of RSV-infection and meningitis due to Hib and meningococci in England and Wales during 1989-1990 [Anonymous, 1991; Jones and Kaczmarski, 1991;

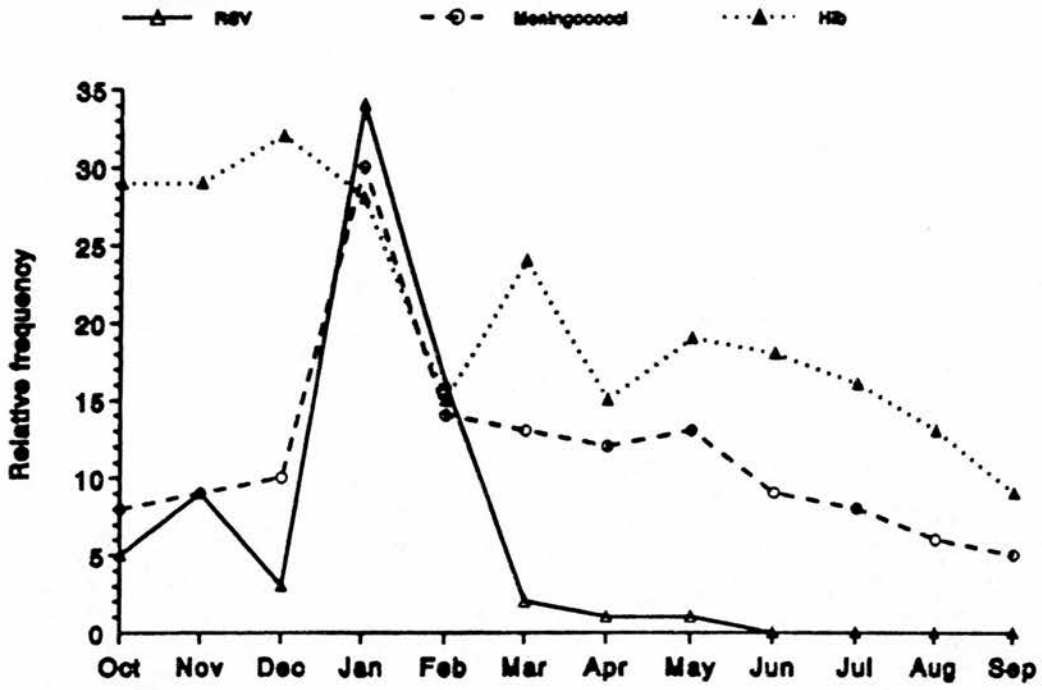


Figure 1.2. Seasonal variations in incidence of RSV-infection and meningitis due to meningococci and Hib in England and Wales during 1989-1990.

Nazareth *et al.*, 1992].

Respiratory syncytial virus belongs to the genus Pneumovirus in the family of Paramyxoviridae. It is an enveloped, large, negative stranded RNA virus, heterogeneous in shape and size. The virus particles are pleiomorphic of approximately 80-350 nm in diameter or filaments of up to 10um in length. The virions assemble at circumscribed regions on the plasma membrane of the infected cell and mature by budding during which intracellular nucleocapsid is packaged within an envelope that is derived from the host cell membrane [Bachi, 1988]. The envelope contains spike-like projections spaced at 6-10nm intervals composed of the major structural glycoproteins G and F which mediate viral attachment (G) [Levine *et al.*, 1987] and fusion and penetration (F) [Walsh and Hruska, 1983]. These glycoproteins are transported to the cell membrane and inserted in the lipid bilayer [Satake *et al.*, 1985; Walsh *et al.*, 1989]. (See section 7.1 for details of viral glycoproteins).

Two antigenically distinct types of RSV, designated A and B subgroups based on variation in G glycoprotein of RSV have been recognized [Anderson *et al.*, 1985; Johnson *et al.*, 1987]. The antigenic variations are shown to have significant effect on the epidemiology of the disease. Antigenic characterization of RSV in 483 isolates in the USA and Canada obtained during 1985/1986 indicated that outbreaks of RSV infection were not nationwide phenomena since small sections of the community were affected by different groups of the pathogen. [Anderson *et al.*, 1991].

A study involving group typing of antigens of RSV-positive nasopharyngeal aspirates from patients indicated a regular alternation in prevalence of the two subgroups. It was also shown that the infants under 6 months were more resistant to re-infection with the homologous than the heterologous virus. Children between 6 and 12 months

were able to produce significant amounts of antibodies to G glycoprotein [Waris, 1991].

The role of antibodies in protection from RSV infection is variable. Specific antibodies do not provide complete protection in adults. Hall *et al.* [1991] investigated re-infections in 15 adults who had a history of natural infection by RSV and who were challenged with the virus. Seven of them became reinfected at the first challenge given at 2 months after the natural infection, and 2/3 were infected by 8 months during which they were challenged at the 2nd, 4th and 8th month. Maternal antibodies (IgG) persisting in infants between 6 weeks to 5 months do not confer protection [Gardner, 1973]. Higher serum antibody levels in infants are, however, found to be associated with upper respiratory infection rather than lower respiratory infection indicating that IgG might protect the lungs [Kasel *et al.*, 1987-1988]. The presence of antibodies in nasal secretions in adult volunteers was associated with reduced virus shedding and upper rather than lower respiratory infection following RSV challenge [Mills *et al.*, 1971]. Appropriate cytotoxic T-lymphocyte responses were associated with decreased severity of RSV infection in volunteers [Isaacs *et al.*, 1990]

1.7. Aims of the study

As viral infections have been proposed to contribute to susceptibility to bacterial meningitis, the objectives of the study were:

1-to determine the relationship of secretor status and respiratory viral diseases;

2-to test the hypothesis that RSV infected cells bind more of the bacteria that cause meningitis than uninfected cells;

3-if binding to RSV-infected cells was increased, to attempt to identify cellular

surface component(s) associated with enhancement of bacterial binding.

Chapter 2

General Materials and Methods

2.1. Buffers and Media

All chemicals were of analytical grade and were obtained from BDH Chemicals Ltd., UK unless otherwise indicated.

2.1.1. Dulbecco's phosphate-buffered saline, solution A (DPBS)

DPBS consisted of sodium chloride (136 mM), potassium chloride (2.6 mM), sodium hydrogen phosphate (8.1 mM), potassium dihydrogen phosphate (1.47 mM), aqueous phenol red (0.2%) at pH 7.3.

2.1.2. Phosphate-buffered saline (PBS)

PBS was composed of 0.01 M phosphate buffer pH 7.2 with 0.15 M NaCl.

2.1.3. Virus transport medium

Virus transport medium consisted of 10.8 g CMRL powder (Gibco), 4.8 g HEPES, 1.0 g $NaHCO_3$, 100 000 unit benzyl penicillin and 40 mg gentamicin in 1 litre of sterile water.

2.2. Reagents used in enzyme linked immunosorbent assays (ELISA) for determination of secretor status

Following reagents were used in ELISA.

2.2.1. Coating buffer (CB)

CB consisted of sodium carbonate (15 mM), sodium bicarbonate (35 mM) and sodium azide (3 mM) (pH 9.6).

2.2.2. Washing buffer

Washing buffer was prepared by adding 0.1% w/v bovine serum albumin (BSA) and 0.05% (v/v) Tween-20 to PBS (pH 7.2). The buffer was used for all washing procedures during the assay.

2.2.3. Blocking buffer (BB)

BB was prepared by adding 1% (w/v) bovine serum albumin to PBS.

2.2.4. Substrate solution

O-phenylenediamine 40 mg in 100 ml of 0.1 M phosphate citrate buffer (0.1 M sodium hydrogen phosphate, 0.1 M citric acid, at pH 5) was used as substrate for the enzyme conjugated to one of the reagents. The substrate was activated with 40 ul of 30% hydrogen peroxide (v/v) immediately before use.

2.3. Preparation of Sepharose beads for purification of blood group substances

2.3.1. Solutions used

2.3.1.1. Coupling buffer

Coupling buffer contained sodium bicarbonate (0.1 M) and sodium chloride (0.5 M) at pH 9.

2.3.1.2. Acetate buffer

Acetate buffer was composed of sodium acetate (0.1 M) and sodium chloride (1.0 M). The solution was adjusted to pH 4 by adding acetic acid.

2.3.1.3. Carbonate buffer

Carbonate buffer was composed of sodium hydrogen carbonate (0.1 M) and sodium chloride (1.0 M) at pH 8.

2.3.1.4. NET buffer

NET buffer consisted of sodium chloride (0.15 M), ethylenediamine tetra acetic acid EDTA (0.04 M), TRIS (0.04 M), phenylmethylsulphonyl fluoride (PSMF) (0.02 mM) and 0.03% sodium azide at pH 7.

2.3.1.5. Eluting buffer

Elution buffer consisted of 1 M acetic acid.

2.3.2. Antibody purification

Synsorb beads (Chembiomed Ltd., Edmonton, Alberta, Canada) with synthetic carbohydrate determinants, Le^a and Le^b, linked to inert particles were used to purify corresponding antibodies. One gram of the appropriate Synsorb beads was mixed overnight at 4^0C with 5 ml of hybridoma culture supernatant containing mouse monoclonal anti-Le^a (LM112/161) or anti-Le^b (LM129/81) antibodies (provided by Dr RH Fraser, Glasgow and West Scotland Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire, UK). The beads were washed twice with PBS by centrifugation at 50 g for 5 min. The antibodies were eluted by mixing 5 ml of 2%

ammonia in saline (pH 11) with the beads for 15 min at room temperature. The beads were stored in 70% v/v ethanol at 4 ^{0}C for further use.

2.3.3. Coupling of proteins to Sepharose beads

Cyanogen bromide activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden) (0.3 G) were washed in 10^{-3} N hydrochloric acid for 20-30 min and centrifuged at 50 g for 10 min. Samples (5 ml) containing antibodies to Le^a and Le^b antigens eluted from synsorb beads (2.3.2) were freeze-dried to remove ammonia. The antibodies were reconstituted in 5 ml of coupling buffer. Ulex europaeus lectin (UEAI) (Sigma, Pool, Dorset, UK) (1 mg) was dissolved in 5 ml of coupling buffer. These solutions were continuously mixed with Sepharose beads overnight at 4^{0}C. The beads were washed 3 times with NET buffer. Unbound active sites were blocked by 1 M ethanolamine (pH 8) for 2 hr. The beads were subjected to three cycles of washings with acetate buffer and carbonate buffer alternately for removal of non-covalently bound proteins. These preparations were used in purification of blood group antigens from saliva.

2.4. Maintenance of HEp-2 cell line

2.4.1. Media used

2.4.1.1. Cell Growth Medium (GM)

GM consisted of Eagle's minimal essential medium (Gibco) supplemented with foetal calf serum (FCS) (Gibco) (10%), $NaHCO_3$ (0.85 g/l), L-Glutamine (2mM), streptomycin (200 ug/l) and penicillin (100 IU/ml). The pH of the final preparation was adjusted to 7.4 with 1 N NaOH.

2.4.1.2. Cell Maintenance Medium (MM)

MM consisted of the same constituents as GM except the quantity of FCS was

reduced to 1%. In some assays involving live bacteria, MM without antibiotics was used. The medium for cells kept in 5% CO_2 in air contained 2 g/l of sodium bicarbonate.

2.4.2. Method

HEp-2 cell line (Flow Laboratories) was used in the studies involving RSV infection of the cells and in bacterial binding assays. The cell line consists of transformed epithelial cells originating from a human laryngeal carcinoma. The cells between passages 400 to 450 were used in the assays. Following two rinses with DPBS, a solution of 0.05% trypsin and 0.02% EDTA (Cell Culture Products) (2ml for a 75 cm^2 flask) was applied to monolayers of the cells for 2-5 min at 37^0C to prepare a suspension for the next passage. The cells used in bacterial binding studies were suspended by applying 1 ml EDTA (0.5%) to monolayers in 25 cm^2 flask for 5-10 min at 37^0C. The effect of the trypsin/EDTA or EDTA on the cells was terminated by suspending the cells in GM or MM. Non-viable cells were detected by preparing a dilution (1/10) of the cell suspension in 0.5% trypan blue in physiological saline (Northumria Biological, UK) which stained these cells. Viable cells (unstained) were counted by light microscopy using an improved Neubauer counting chamber. Cells from freshly confluent monolayers were suspended in 1 ml of 10% dimethyl sulphoxide (DMSO) (Sigma) in GM and stored in liquid nitrogen following gradual cooling to -70^0C. Frozen cells were resuscitated by rapid thawing at 37^0C for further use.

2.5. Preparation and standardization of respiratory syncytial virus (RSV)

2.5.1. Growth of viruses

Edinburgh strain of RSV [Ogilvie *et al.*, 1981] was used in these assays. Stocks of

virus from 5th to 7th passages were prepared as follows.

One-day-old sub-confluent monolayers of HEp-2 cells were infected with RSV at multiplicity of infection (M.O.I.) of 2-3 infectious particles per cell for one hr. The monolayers were maintained in MM for 48-72 hr at which time the cells began to fuse due to the RSV-infection. About 4/5 of the medium in the flask was discarded and the flask was frozen at -70^{0}C and then thawed to lyse the cells. The suspension was centrifuged at 700 g for 10 min to get rid of large cell debris. Aliquots of the supernatant were stored at -70^0 C for further use.

2.5.2. Plaque assay

2.5.2.1. Solutions

Overlay medium (O/L) was prepared by adding methyl cellulose from a stock preparation (3% w/v methyl cellulose in Hanks' salt solution) (25%) and $NaHCO_3$ (to a final of 2 g/l) to MM.

Staining solution was used to examine cell monolayers for syncytia and plaque formation. This solution contained crystal violet (0.13%) and formalin (5% v/v) in normal saline.

2.5.2.2. Method

Cell monolayers were grown in 24-well tissue-culture plates (Costar) for 24 hr. Ten-fold dilutions of the virus suspension were distributed to wells in quadruplicate (200 ul/well) and adsorbed to monolayers for 1 hr at 37^0 C. The supernatant was removed from the wells and 1 ml of overlay medium added to each well. The plates were incubated in 5% CO^2 at 37^{0}C for 3-4 days until syncytia/plaques appeared in the monolayers. The monolayers were fixed and stained with the crystal violet stain for

20 min and washed with tap water. The monolayers were examined by inverted light microscopy for syncytia and plaques.

2.5.3. Detection of RSV infected cells

Samples of cells in monolayers and suspension were examined for the proportion of RSV-infected cells by fluorescence microscopy and flow cytometry.

2.5.3.1. Fluorescence microscopy

One day old monolayers of HEp-2 cells on glass coverslips (13 mm) were infected with different M.O.I. of RSV for 24 hr. Monolayers were fixed with chilled acetone (BDH) for 5 min and reacted with mouse monoclonal antibody to F glycoprotein of RSV (1/100) (20 ul/coverslip) (kindly supplied by Prof. P.J. Watt, Southampton University) for 30 min at 37^0C. After washing 3 times with PBS, 20 ul of FITC-labelled anti-mouse antibody (1/50) (Fab fragment, absorbed with human serum protein) (Sigma) were added to each coverslip for 30 min. The monolayers were washed 3 times, dried and mounted with 50% glycerol in PBS. They were examined by ultraviolet microscopy for the proportion of fluorescent cells present.

2.5.3.2. Flow cytometry

Monolayers of HEp-2 cells were infected with different M.O.I. of RSV for 24 hr. The cells were suspended as described in section 2.4.2. The cells in suspension (200 ul) (1 X 10^6/ml MM) were reacted with the same antibodies as above. The final concentration of anti-F antibody was 1/100 and that for FITC-labelled anti-mouse immunoglobulin antibody was 1/200. The cells were washed 3 times with PBS by centrifugation at 680 g for 7 min. The cells were suspended in 200 ul PBS and fixed with 100 ul 1% buffered paraformaldehyde. The proportions of fluorescent cells and

mean channel fluorescence of the cells were determined by flow cytometry (see section 2.7).

2.6. Bacteria

Meningococci (3 strains), *H. influenzae* (5 strains) (Tables 2.1 and 2.2) and *Staph. aureus* (1 strain) were used in the assays. Hib isolates from Icelandic children were kindly provided by Dr. K. Jonsdottir, University of Iceland. Serogroups, serotypes and subtypes of meningococcal isolates were determined by Dr. R.J. Fallon, Meningococcal Reference Laboratory, Scotland.

2.6.1. Media

Modified New York City medium (MNYC)

This medium was prepared as described by Young [1978]. GC medium base (Difco, UK) was supplemented with 10% (w/v) horse blood lysed by saponin (0.5% w/v), yeast dialysate (2.5% w/v), glucose (0.1% w/v), lincomycin (1 ug/ml), colistin (6 ug/ml), amphotericin B (1 ug/ml) and trimethoprim lactate (6.5 ug/ml).

Boiled blood agar (BBA) was prepared by heating sterile blood (10% v/v) in nutrient agar.

GC-plus medium contained GC agar supplemented with proteose peptone No. 3 (15 gm/l), corn starch (1 gm/l) dibasic potassium phosphate (4 gm/l), monobasic potassium phosphate (1 gm/l) and sodium chloride (5 gm/l).

2.6.2. Maintenance and storage of bacteria

Bacterial cultures were prepared by reconstituting lyophilized strains in distilled

Table 2.1. Isolates of *Neisseria meningitis* examined

Serogroup	Serotype	Subtype	Source
C	2b	P1.2	patient
NG	-	P1.9	carrier
Y*	14	P1.2	Carrier

* This strain was used in both pilate and non-pilate forms. NG non-groupable

Table 2.2. Isolates of type b *Haemophilus influenzae* examined

Strain No.	Enzyme type	Biotype	OMP type	LPS type	Source
14	--	--	--	--	Edinburgh
20	4b	1	NT	1	Reykjavik
21	1	1	2	9	Reykjavik
25	12	1	1	1	Reykjavik
29	1	--	--	--	Reykjavik

NT - non-typable

water and plating them on appropriate media. Meningococci were grown on MNYC medium for most of the binding assays and *H.influenzae* on BBA. For some experiments, meningococci were grown on BBA or GC medium to examine the effect of the medium on bacterial binding. Meningococci for the latter part of the study were grown on BBA. An overnight growth of meningococci and 48 hr culture of *H. influenzae* at 37^0 C in 5% CO_2 were used in the assays. The bacteria were suspended in soya broth containing dextrose (0.25%) (DIFCO) and stored at -20^0C. Subcultures were prepared from these frozen stocks.

The bacteria were harvested, washed 3 times by centrifugation at 2500 g for 10 min. They were resuspended in MM without antibiotics for use in the binding assays with cells in monolayers or were labelled with a fluorescent dye, washed 3 times and suspended in MM without antibiotics for use in assays with cells in suspension (see below).

2.6.3. Determination of bacterial concentration

For each strain, the bacterial concentration was determined by optical density (OD) at 540 nm and by light microscopy with a Thoma counting chamber. The range of readings of OD having a linear relationship with the counts for each strain is given in Fig 2.1. The bacterial suspensions were adjusted to provide a range of ratios of bacteria per cell for use in the assays.

2.6.4. Labelling of bacteria

Fluorescein isothiocyanate (FITC) (Sigma) was used for labelling the bacteria in most assays. The solution was prepared as described by Rahat [1990]. FITC (400 ug) was dissolved in 1ml of buffer containing sodium carbonate (0.05 M) and sodium chloride (0.1 M) at pH 9.2.

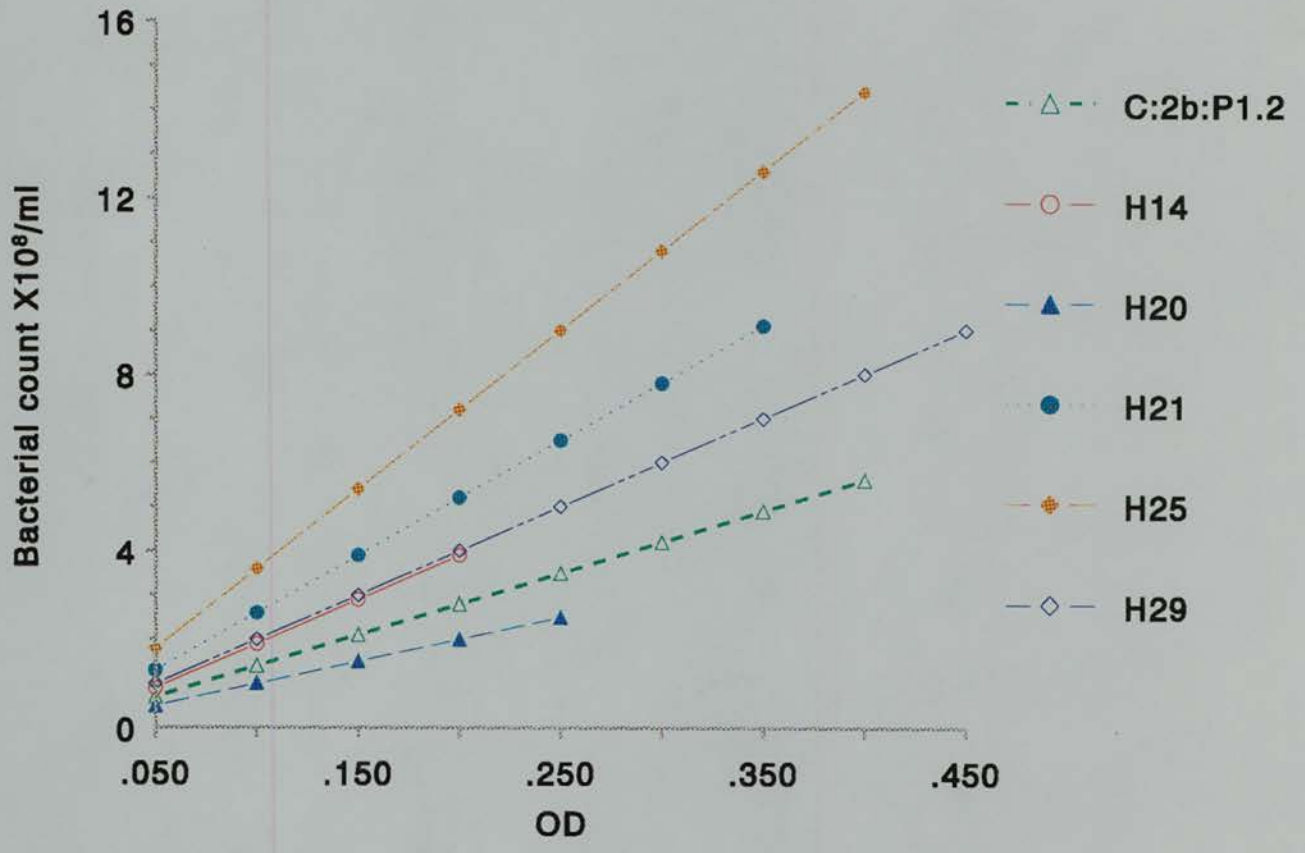


Figure 2.1. Optical density (OD) measurements and bacterial counts determined by light microscopy.

The washed bacterial pellet from two culture plates was suspended in 2 ml of FITC solution by gentle shaking at 37^0C for 20 min. The FITC-labelled bacteria were washed three times with DPBS and resuspended in MM without antibiotics. The bacterial concentration was adjusted as described above.

Some aliquots of bacteria were also labelled with rhodamine 123 (Eastman Organic Chemical, Rochester) to compare the efficiency of the two fluorescent dyes. Rhodamine was dissolved in distilled water (1mg/ml) and diluted in equal volume of MM before use. Bacterial pellets from 2 plates were incubated with 2 ml of rhodamine solution for 30 min or 1 hr (see 5.3.3.1 for detail).

2.7. Detection of bacterial binding by flow cytometry

Flow cytometry involves sensing particles as they move in a liquid stream past a laser beam. Patterns of scattered light carrying information about size, granularity and fluorescence on particles are gathered by sensors appropriately placed around the stream. The information is displayed in histograms having various assigned parameters, e.g., size. Setting up of the system involves placing a cursor in a histogram (see below) to demarcate fluorescent populations from non-fluorescent ones and adjusting the photosensitivity of the machine.

The cell samples were analysed with an EPICS-C (Coulter Electronic, Luton, UK) equipped with a 5 watt laser with a power output of 200mWat 488 nm. A bitmap was drawn to enclose the main population of the cells from a display on a histogram showing the signals of size and granularity of the cells. The percentage of the fluorescent cells in the main population was obtained from a one-parameter histogram (H-%) with a logarithmic scale. The cursor was placed at channel 55 of H-%. The photosensitivity of the machine was adjusted in such a way that about 2% of the cells in non-fluorescent samples used as control for each experiment were assessed as

fluorescent with reference to this channel. The mean values of fluorescence from positive cells were acquired from a one-parameter histogram measuring fluorescence on a linear scale.

The data obtained from test samples recorded on H-% histograms were compared with that from a control sample for each experiment using the immunoanalysis program (Coulter, UK). This computer programme subtracts the values of control population from the test population at each channel. For each sample, a binding index (BI) was calculated by multiplying the percentage of fluorescent cells obtained from the immunoanalysis and the mean of fluorescence.

Chapter 3

Secretor status and viral infection

3.1. Introduction

Susceptibility to a variety of bacterial and superficial fungal infections is associated with the genetically controlled inability of individuals to secrete the water soluble form of their ABO blood group antigens into body fluid (non-secretion). Non-secretors are also over-represented among patients with some autoimmune diseases for which infectious triggers have been proposed (Table 1.3). Associations between ABO blood groups and viral infections have also been investigated. McDonald and Zuckerman [1962] found a substantial relative excess of blood group O patients and a corresponding deficiency of blood group A patients among 2,000 army personnel with infection by H2N2 strain of influenza A virus during 1956 to 1961. An opposite trend of blood groups for an association with infection due to adenovirus was noted in the group. An association between infection with another strain (H3N2) of influenza A virus and blood group B was also reported [Mackenzie and Fimmel, 1978].

Repeated exposure of a population to a virus will result in development of specific immunity in the individuals. This might obscure any effect associated with blood groups to viral infections in non-immune individuals. Potter [1969] found no difference in the incidence of infection with different strains of influenza virus and adenovirus among people of different blood groups in a population which was repeatedly exposed to epidemics of these viral infections. Evans [1975] found no association between ABO blood groups and infection due to influenza A and B

viruses, parainfluenza virus or adenovirus in a prospective study of 925 recruits.

If viral infections can contribute to susceptibility to meningococcal disease, this might be due to non-secretors being at increased risk of viral infections. So far, associations between secretor status and viral infections have not been studied. In this study, the hypothesis that non-secretors might also be at increased risk of viral infection was examined.

Because the quantities of material available from patients with viral diseases were too small to determine secretor status by the usual haemagglutination inhibition method [Mollison,1979], an enzyme linked immunosorbent assay (ELISA) was developed to detect Lewis blood group antigens in the specimens. Non-secretors express only Lewis^a (Le^a) and secretors express Lewis^b (Le^b) and variable amounts of Le^a on their red blood cells and in their body fluids (Table 1.1).

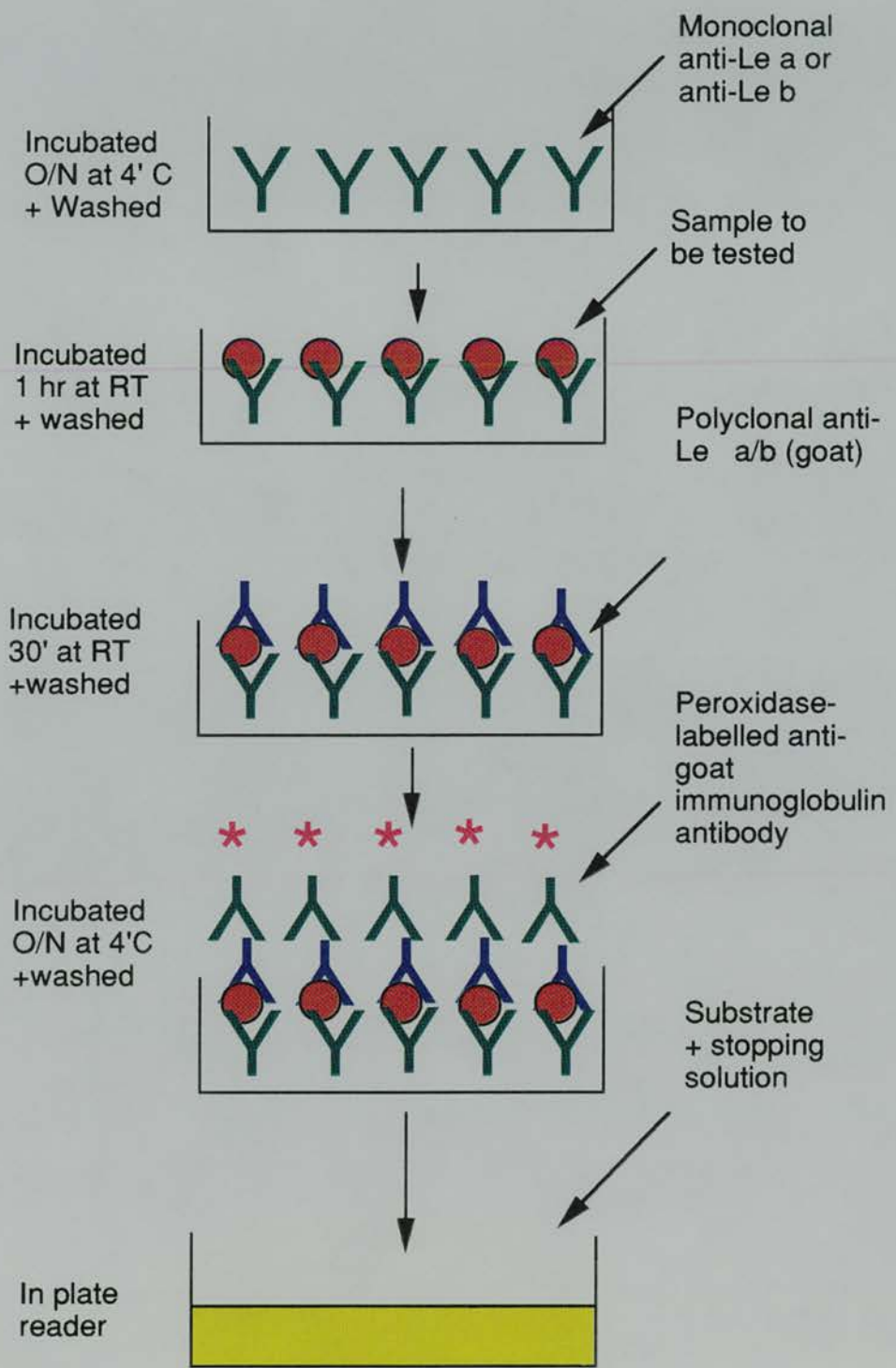
3.2. Materials and methods

3.2.1. ELISA for Lewis antigens

Le^a and Le^b blood group antigens were detected in body fluids by an ELISA (Fig 3.1). Wells of polystyrene microtiter plates (Dynatech, Billinghurst, Sussex) were coated overnight at 4^{0}C with 100 ul of monoclonal anti-Le^a diluted 1/25 or monoclonal anti-Le^b diluted 1/20 in coating buffer (2.3.2). All further procedures were carried out at room temprature except when stated otherwise. The wells were washed 3 times with washing buffer and blocked with 150 ul of blocking buffer for 15 min. The buffer was removed and the wells were washed 3 times.

Dilutions of saliva from known secretors and non-secretors were used in each plate as controls. The test wells contained 100 ul of the specimens (saliva, nasal washings or respiratory secretions) which had been boiled for 30 min to inactivate enzymes,

Figure 3.1. ELISA for detection of Lewis antigens.



bacteria or viruses. Samples of saliva were diluted 1/100 in blocking buffer for detection of Le^a, and 1/20 for detection of Le^b. The more dilute nasal washings or respiratory specimens were diluted 1/10 in blocking buffer to detect Le^a antigens but were used undiluted to detect Le^b antigens.

After incubation for 60 min, the wells were washed 3 times and 100 ul of polyclonal goat anti-Le^a antibody (Behring, Marburg, West Germany) (1/500 in blocking buffer) or 100 ul of anti-Le^b antibody (Behring) (1/250) were added to the wells of the appropriate plates. After 30 min incubation, the wells were washed 3 times, and 100 ul of horseradish peroxidase-conjugated donkey anti-goat immunoglobulin (Scottish Antibody Production Unit, Carluke, Lanarkshire) (1/250 in blocking buffer) were added. After overnight incubation at 4^0C, the plates were washed three times and 100 ul of activated substrate solution were added to each well. The colour was allowed to develop for 10-15 min, and the reaction was stopped by adding 50 ul of stopping solution to each well.

Absorbance at 490 nm was measured with an ELISA plate reader (MR 700) (Dynatech). Samples were tested in duplicate and readings were averaged. The averaged reading obtained for the test samples was compared with that of the controls added in the same plate. Values equal to or above that of control were considered to be positive. The subjects from whom the samples were obtained were classified as non-secretors if only Le^a was detected or as secretors if Le^b or both Le^a and Le^b antigens were detected.

3.2.2. Samples and controls

To assess the method, the results obtained by the ELISA were compared with those obtained by a haemagglutination inhibition assay (HAI) for 1155 saliva specimens collected during the Stonehouse study [Blackwell *et al.*, 1989c]. Nasal secretions

were collected from 26 members of staff whose secretor status and Lewis blood group antigens had been previously determined. Secretions were collected with cotton wool swabs and inoculated into virus transport medium. The transport medium was assayed for presence of Lewis antigens. Nasal washings (n=872) obtained from volunteers as part of other studies at the Medical Research Council's Common Cold Unit were also tested to determine if the Lewis antigens could be detected in diluted nasal secretions. They were originally obtained from the volunteers for assessment of secretory antibodies and stored at -20^{0}C for 4-5 years.

As there is no association between sex and secretor status or sex and Lewis blood group [Mourant *et al.*, 1976], the distribution of Le^a and Le^b antigens in the local population was determined with red blood cells from 363 women attending antenatal clinics at the Royal Infirmary, Edinburgh, by standard tube agglutination method with the monoclonal anti-Le^a and anti-Le^b antibodies referred to above in 10% Dextran and 2% BSA [Scottish National Blood Transfusion Service]. The results were compared with those of a previous study of the local population in which secretor status of 334 blood donors was determined from saliva by HAI [Kinane *et al.*, 1982].

Specimens (584) sent to the Regional Virus Laboratory, mainly from hospitalized patients (age group 1 month to 90 years) with symptoms of viral disease were used in this study. These were aspirated nasal or respiratory secretions or throat swabs inoculated into virus transport medium (2.1.3) which had been stored at -70^{0}C following culture for virus.

The sources of specimens used in this study are summarized in Table 3.1. The results for local population and test specimens were compared by X^2 test incorporating Yates' correction factor. Odds ratios and 95% confidence intervals (CI) were calculated by the exact method.

Table 3.1 Source of specimens for determination of Lewis blood group and secretor status

Source	Specimen	No.
Controls		
Local antenatal clinic	Blood	363
Local antenatal clinic	Saliva	334
Stonehouse study	Blood & saliva	1155
MRC Common Cold Unit	Nasal washings	872
Laboratory personnel	Saliva, blood & nasal swabs inoculated into virus transport medium	26
Patient specimens		
Regional Virus Laboratory	Nasal secretions OR Swabs inoculated into virus transport medium	584

3.3. Results

3.3.1. Assessment of the ELISA for detection of Lewis antigens in the body fluids

Le^a, Le^b or both antigens were detected by the ELISA in 1089 of the 1155 (94.3%) saliva specimens for which secretor status had been determined by HAI. The results of ELISA for Lewis antigens and HAI agreed for 1058 (97%) of 1089 Lewis positive specimens, 796 Le^b antigen positive (secretors) and 262 Le^a antigen positive (non-secretors). The results for the two assays disagreed for 31 samples; 27 were positive for Le^a only (non-secretors) by the ELISA but secretors by HAI and 4 specimens were Le^b positive (secretors) by the ELISA but non-secretors by HAI. Lewis antigens could not be detected by the ELISA in 66 specimens (5.7%): 30 were from secretors and 36 from non-secretors as determined by HAI. Lewis phenotype determined by agglutination of red cells for 124 of these donors agreed with the results of the ELISA for 96% of those tested. The ELISA correctly identified the Lewis antigen present in nasal secretions of all 26 laboratory staff. Among the 872 nasal washings obtained from the Common Cold Unit, Lewis antigens were detected in 854 (97.9%): Le^a antigen in 233 (26.7%); Le^b in 621 (71.2%); and no Lewis antigen in 18 (2.1%). The results do not differ from the distribution of these phenotypes in most northern European populations [Mourant *et al.*, 1976].

3.3.2. Lewis phenotypes of the local population

Among 363 blood specimens from the antenatal clinic (Table 3.1) for which the Lewis antigens were determined, 28% were Le^a and 72% Le^b. These results were not significantly different from the proportion of non-secretors (26.6%) and secretors (73.4%) determined in a previous study by HAI tests of 334 saliva samples from local blood donors [Kinane *et al.*, 1982].

3.3.3. Lewis phenotype of patients with viral illnesses

In 192 of the 584 (33%) patients' specimens examined by ELISA, there was not enough Lewis antigen detected for definite classification. There were 81 borderline ELISA readings, and in 111 no antigen was detectable. Among the 192 specimens that were borderline or Lewis-negative, no virus was isolated from 38 (20%). Among the 392 specimens in which either or both Lewis antigens were definitely detected, no virus was isolated from 36 (9%) cases (X^2=12.17, P<0.0005). The proportion of unclassifiable specimens did not vary significantly with respect to isolation of any particular virus. As the specimens giving negative and borderline results for Lewis antigens could not be classified as being from secretors or non-secretors, they were eliminated from further analysis.

Some of the specimens (n=60) which were positive for Le^b and for RSV, rhinovirus or parainfluenza virus (20 from each group) were deliberately freeze-thawed at least twice. They were then examined in the ELISA to investigate the effect of freeze-thawing on the detection of the Lewis antigens. These samples were also tested in the ELISA for H antigen for confirmation of the results.

The repeat-ELISA detected Le^a in some previously Le^a-negative samples. Le^b was detected in fewer samples in the repeat-assay compared with the first test. Not all the samples positive for Le^b also showed H activity (Table 3.2).

Isolation of viruses from Le^a/non-secretors and Le^b/secretors is compared in Table 3.3. Compared with the local population, there was a significantly higher proportion of secretors among subjects from whom the following viruses were isolated: Influenza A virus (P<0.05), rhinovirus (P<0.01), respiratory syncytial virus (RSV) (P<0.0005), and echovirus (P<0.0005). Although 11 of 13 specimens with influenza B virus were secretors, the numbers were too small to be significant. This pattern was not observed for the 67 specimens from which parainfluenza virus was isolated or the 36 specimens from which no virus was isolated. In these two groups the

Table 3.2. Effect of freeze-thawing on detection of Lewis and H antigens in samples of nasopharyngeal washings determined by ELISA

Results	**First Assay**		**Repeat assay**		
	Le^a	**Le^b**	**Le^a**	**Le^b**	**H**
Positive	0	60	16	34	20
Negative	52	0	35	6	16
Borderline	8	0	9	20	24

TABLE 3.3 Lewis phenotype/secretor status of patients and results of virus culture

Specimen source	Virus isolated	Le^{a+b-} non-secretors No.	(%)	Le^{a-b+}/Le^{a+b+} secretors No.	(%)	χ^2	P	Odds Ratio*	(95% CI)
antenatal clinic controls		103	(28)	260	(72)				
Regional Virus Laboratory									
	Influenza (total)	9	(14)	55	(86)	5.74	<0.025	2.42	(1.13-5.77)
	A	7	(14)	44	(86)	4.92	<0.05	2.49	(1.06-6.76)
	B	2	(15)	11	(85)	0.51	>0.05	2.18	(0.46-20.53)
	Parainfluenza	17	(25)	50	(75)	0.13	>0.05	1.17	(0.63-2.26)
	RSV	12	(11)	97	(89)	12.77	<0.0005	3.20	(1.66-6.67)
	Rhinovirus	9	(12)	63	(88)	7.11	<0.01	2.77	(1.31-6.57)
	ECHO	0	(0)	44	(100)	15.25	<0.0005		(4.41-)
	no virus	9	(25)	27	(75)	0.06	>0.05	1.19	(0.52-2.97)

* Odds ratio compared with local controls.

proportions that were Le^b antigen positive (secretors) and Le^a antigen positive (non-secretors) were similar to that of the local population.

3.4. Discussion

3.4.1. Validation of method

Determination of Lewis phenotype is a good control for the HAI for ABO antigens which has been the standard method for determination of secretor status. Agreement between the Lewis phenotype and HAI for 1089 specimens was 97%. "False secretors", Le^a type by ELISA but secretors by HAI predominated among mismatched pairs (27/31, 87%). Results of previous studies indicate that these results are most likely due to contamination of saliva by blood owing to poor oral hygiene or periodontal disease among these subjects [Blackwell *et al.*, 1989c]. Dilution of small samples to provide enough material for the haemagglutination inhibition test is probably the source of the small proportion (0.4%) of "false" non-secretors who are of Le^b phenotype but non-secretors by HAI.

The ELISA method eliminates the problems of contamination of non-secretors' saliva by red cells and it can be carried out on smaller volumes than those needed for HAI. The method detected Lewis antigens in 854/872 (98%) specimens from the Common Cold Unit, indicating that it can be used to assess these antigens in diluted nasal secretions

3.4.2. Results from patients with viral disease

The nasal washings from volunteers at the Common Cold Unit were originally collected for determining secretory antibody titres and were frozen soon after collection which would preserve the Lewis antigens; analysis of the results found the

expected (2%) proportion of specimens negative for Lewis antigen. The high proportion of specimens from patients in hospital with viral illness for which borderline readings were obtained or no Lewis antigen was detected might be due, in part, to collection techniques, time taken for transportation and processing, and repeated freeze-thawing of the specimens.

There was a significantly higher proportion of unclassifiable specimens from which no virus was isolated (20%) compared with the proportion of specimens in which Lewis antigens were definitely detected but from which no virus was isolated (9%) ($P < 0.0005$). The proportion of unclassifiable specimens was not greater among the very young age group (<24 months) from whom nasopharyngeal secretions are usually obtained. Although Lewis antigens were correctly identified in all the samples of transport medium inoculated with nasal swabs from 26 laboratory staff, secretions provide a larger quantity of material for isolating virus and detection of the Lewis antigens.

Repeated freeze-thawing could also affect the quality of samples due to degradation of the blood group antigens (Table 3.2). Blood group antigens cannot be reliably detected in saliva kept overnight at room temperature (personal communication). The results obtained with these specimens might reflect degradation of the antigens due to delays in their transportation to the laboratory.

The terminal fucose of Le^b added by the transferase encoded by the secretor gene is also recognized as the H determinant in the ELISA for detection of H. The assay for H determinant is different from the ELISA for Le^b; it is more sensitive and the antigens in the samples are directly coated onto the surface of the wells instead of being captured by antibodies as in the ELISA for Lewis antigens. H antigen was detected less frequently in specimens than Le^b antigen in the repeat assays (Table 3.2). This might reflect some enzymatic changes in the specimens due to repeated

freeze-thawing affecting the coating of the antigens. The possible conversion of specimens from Le^b to Le^a by freeze-thawing cannot adversely affect the results in this study since Le^b positive specimens were over-represented in this data.

Previous studies found non-secretion of ABH antigens to be associated with various bacterial diseases and superficial yeast infections and with carriage of some pathogenic bacteria or yeasts [Burford-Mason *et al*, 1988; Blackwell *et al*, 1989b]. In contrast, the findings in the present study indicate that secretors were over-represented among the hospitalized patients with respiratory illness due to influenza A virus, rhinovirus, or RSV. Secretors were also significantly over-represented among those from whom echoviruses were isolated; these patients, however, had various illnesses including meninigits, fever, and vomiting. An increase in the proportion of secretors was not associated with isolation of parainfluenza virus or with the group of individuals from whom no virus was isolated.

In another study [Blackwell *et al.*, 1991], a similar increase in the proportion of secretors (88%) was identified in a group of 26 individuals who had acquired human immunodeficiency virus (HIV) by heterosexual intercourse compared with 70% secretors among 191 patients who acquired the infection by intravenous injections or homosexual intercourse. In contrast, there were only 46% secretors in a group of 28 HIV negative individuals who were at risk but who had not acquired the virus through heterosexual intercourse. This finding suggests that the heterosexual secretors are at a higher risk of the infection and that non-secretion is protective.

The hypothesis that viral respiratory tract infections might be associated with non-secretors was disproved. The following hypotheses were proposed to explain the associations between secretion and respiratory viral disease.

(1) There is evidence that Le^a antigen present in greater amounts on epithelial

surfaces of non-secretors might be one of the receptors for *N. meningitidis* [Rahat, 1990]. Binding of staphylococci was significantly correlated with the amount of Le^a antigen expressed on buccal epithelial cells of secretors and non-secretors [Saadi *et al.*, submitted for publication]. The association of viral disease with secretors suggests that Le^b antigen present only in secretors or the higher levels of H (type 1 plus type 2) in secretors might be receptors for the viruses. H-type 1 and Le^b antigens are present in the secretions as well as on the surface of the epithelial cells. The antigens in the secretions might inhibit the viral attachment to the cells, but, as these molecules can still adsorb to cells, the net effect of the virus-blood group antigen interaction might be an enhancement of the attachment.

(2) There is some evidence that galactosyltransferases are present on the surface of epithelial cells [Davis *et al.*, 1984]. Mandrell *et al.* [1988] demonstrated structures with blood group specificities on bacteria of *Neisseria* species and proposed a role for glycosylation of these structures catalysed by cell surface enzymes in bacterial adhesion. Presence of comparable enzymes on cell surfaces that interact with the viral proteins might be a susceptibility factor.

(3) Arendrup *et al.* [1991] demonstrated that antibodies to blood group A antigen inhibited HIV grown in lymphocytes from blood group A donors but not from donors of blood group B or O. Although the immune interaction of HIV from hosts with other blood groups is not known, the finding suggests that the shed viruses can acquire host glycoproteins. This is further supported by presence of high amounts of HLA-DR antigens in the envelope of HIV grown in HLA-DR-positive cells [Schols *et al.*, 1992]. Presence of natural antibodies such as anti-Le^b in non-secretors might be protective against the virus infection passed from a secretor contact. An association between ABO blood groups and HIV has not been found.

(4) The association of secretors with viral respiratory tract infections might be related

to the severity of the illness in these patients since the samples tested in the study were from hospitalized patients. The following possibilities are considered based on this observation:

(a) Viruses uniquely depend upon cellular metabolic functions. Addition of sugars to glycoproteins of viral origin is catalysed by cellular enzymes. Lack of glycosylation of viral proteins might result in altered infectivity of the viruses. A single mutation in avian H5N2 influenza virus associated with loss of a specific oligosaccharide side chain is responsible for increased virulence of the virus [Kawaoka *et al.*, 1984]. Normal bovine and mouse sera contain a lectin, termed b inhibitor, which inhibits the infectivity of H3N1 strain of influenza virus. A single mutation in this strain resulting in loss of a potential glycosylation site was associated with development of resistance of the virus to these sera [Anders *et al.*, 1990]

Lack of sugars might have deleterious effects on the infectivity of viruses. Enzymatic removal of only few very accessible oligosaccharide side chains from RSV results in a 76% to 98% reduction in infectivity [Lambert, 1988]. Inhibition of N- or O-glycosylation reduces the infectivity of a variety of enveloped viruses [Leavitt *et al.*, 1977; reviewed by Grubber and Levine, 1985]. Virus infections have been shown to induce host cell enzyme genes not normally expressed by the cell [Arendrup *et al.*, 1991]. Secretors might be at a particular disadvantage due to the presence of an additional enzyme (a fucosyltransferase from the secretor gene) in their cells. Fucosylation of viral proteins by this enzyme might be critical in viral replication, infectivity or spread of infection. If this is the case, the initial attachment of viruses and subsequent mild infection might not be affected by secretor status. In the study reported here, the viral diseases found to be associated with secretion belonged to a heterogeneous group suggesting factors other than a common receptor for different viruses.

(b) An important factor common to most viral infections is cytolytic immunity. Severe infection in volunteers challenged with RSV appeared to be associated with low CTL response [Isaacs *et al.*, 1990]. Loci encoding proliferation and differentiation molecules for immune cells, CD23, CD33, a protein from the LYL1 locus, and the secretor genes are all located on chromosome 19. A transcription factor from the TCF3 locus, an enhancer of kappa chain production, is also located on this chromosome [Ropers and Pericak-Vance, 1990]. A linkage between these loci and the locus for the secretor gene has not been described. The only known linkage between the secretor gene and genes involved in the immune system is that with the gene for the third component of complement [Elberg *et al.*, 1983]. If a genetic linkage between the secretor gene and the genes involved in the cytotoxic T-lymphocyte response existed, this might be responsible for the association between secretors and viral infections.

Inhibition of respiratory syncytial virus infection by purified blood group antigens

4.1. Introduction

Cellular receptors for respiratory syncytial virus (RSV) have not so far been identified [Lentz, 1990]. Secretion of ABH blood group antigens has been significantly associated with disease caused by RSV ($P<0.0005$) (chapter 3). Epithelial cells of secretors bind significantly more *Ulex Europeus* (UEAI) lectin than cells of non-secretors [Rahat *et al.*, 1990]. The lectin binds to fucose-containing molecules and fucose is the immunodominant sugar found on H-type 1 and 2 and Le^b. While type 2 ABH antigens are part of the structure of the cells, it was not known whether HEp-2 cells used for studies of viral infection express Lewis and H blood group antigens. In this chapter, the hypothesis that Le^b and/or H-type 1 antigen might be the receptors for the virus was tested. If so, the virus attachment might be inhibited by the antigen(s) in soluble form.

4.2. Materials and methods

4.2.1. Detection of H and Lewis antigens on HEp-2 cells

4.2.1.1. Fluorescence microscopy

Monolayers of HEp-2 cells grown on cover slips were fixed with chilled acetone (BDH) for 5 min and washed 3 times with PBS. After drying, 20 ul of monoclonal antibodies, anti-Le^a, anti-Le^b (2.3.2) and anti-H_2 (Lm92/90) [Machie *et al.*, 1984],

neat or diluted 1/5 in PBS, were added to the coverslips for 30 min at 37^0C. After washing 3 times, 20 ul of fluorescein isothiocynate (FITC)-labelled anti-mouse immunoglobulin antibodies (1/50) (Sigma) were added to the coverslips for 30 min at 37^0C. UAEI conjugated with FITC (Sigma) (50 ug/ml) was added in 20 ul volume to some coverslips under the same conditions. The coverslips were washed 3 times with PBS and dried. They were mounted with 50% glycerol in PBS and examined by ultraviolet microscopy and the fluorescence of the cells scored.

4.2.1.2. Determination of blood group antigens by Flow cytometery

Suspensions of HEp-2 cells were prepared at $1X10^9$ cells/ml in MM. These heavy suspensions were kept at 37^0C for 2 hr to give time for the cells to secrete and adsorb blood group antigens before proceeding further. The count was then adjusted to $1x10^6$/ml. The cells were first incubated with the neat, 1/5 or 1/10 dilutions of anti-Le^a, anti-Le^b or anti-H_2 for 30 min at 37^0C. The binding of the antibodies to the cells was assessed by incubating the cells with FITC-conjugated anti-mouse immunoglobulin antibodies (Sigma) (1/200). FITC-conjugated UEAI (20 ug/ml) was used for some of the samples to detect the total amount of H antigens (H-type 1 and 2) on the cells. HEp-2 cells were compared with buccal epithelial cells (BEC) of secretors for presence of Le^b and H and with BEC of non-secretors for presence Le^a.

In a separate set of experiments, a direct immunofluorescence technique was used to detect the antigens. Suspensions of HEp-2 cells prepared as above (200 ul) were incubated with a neat or 1/5 dilution of FITC-labelled monoclonal anti-Le^a, anti-Le^b or anti-H_2 [Rahat, 1990] for 30 min at 37^0C. The cells were washed 3 times with DPBS, suspended in 200 ul DPBS and fixed with 100 ul 1% buffered paraformaldehyde. The samples were analysed on an EPICS 'C' flow cytometer for the percentage of fluorescent cells and the mean fluorescence.

4.2.2. Purification of blood group antigens

Blood group substances with Lewis^a, Lewis^b and H type 1 specificities were purified from saliva.

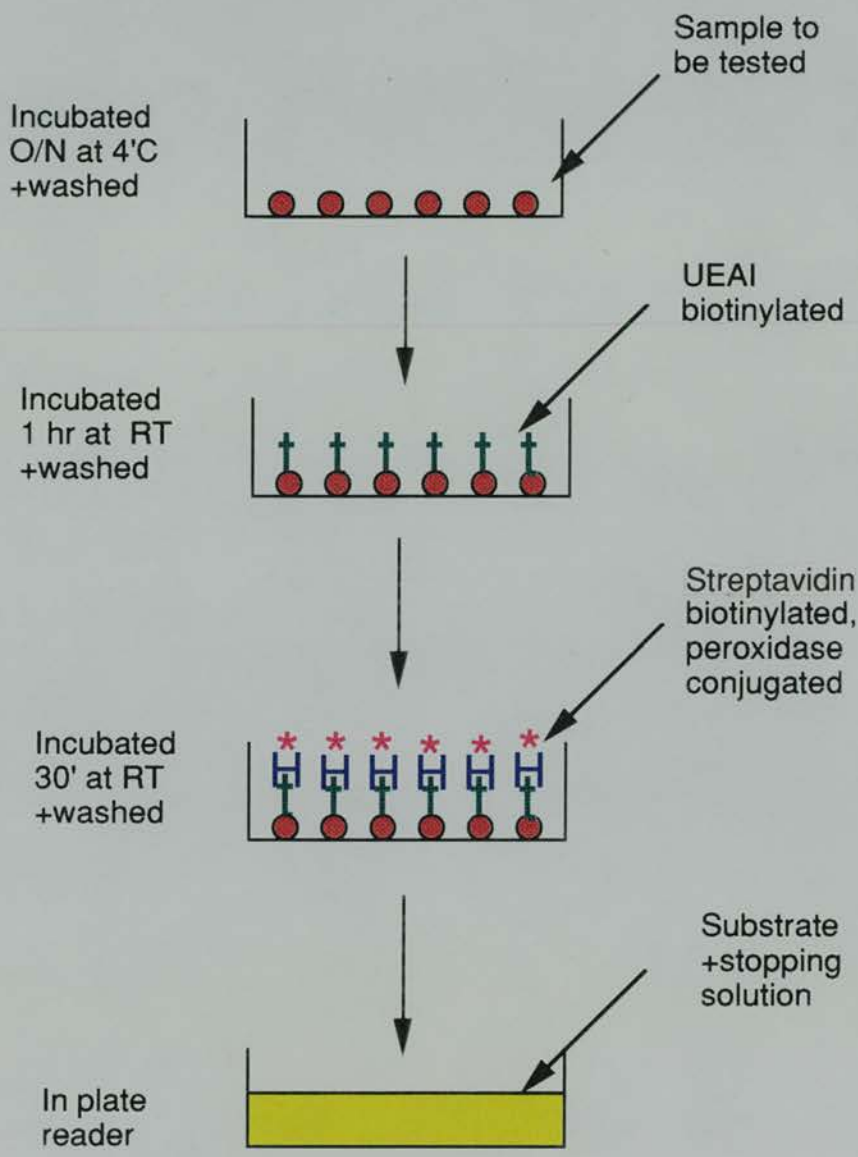
The Method described by Rahat, [1990] was modified to purify blood group antigens from samples of saliva obtained from non-secretors (Lewis^a), secretors (Lewis^b) and Lewis negative, blood group O secretors (H-type 1). The samples (20 ml) were boiled and centrifuged at $1X10^3$ g for 20 min. The supernatant was freeze-dried and reconstituted in 5 ml of distilled water. The reconstituted solutions were added to appropriate Sepharose beads conjugated with monoclonal anti-Le^a, anti-Le^b or UEAI (2.3.3) and incubated overnight at 4^0 C. The beads were washed 3 times with NET buffer by centrfugation at 50 g for 7 min. The antigens were eluted from the beads with 3 ml of 1 M acetic acid added for 10 min. The antigen solutions were dialysed against PBS for 48 hr, sterilized by boiling for 10 min and stored in 200 ul aliquots at -20^0C until used in virus inhibition studies.

4.2.3. ELISA for detection of purified antigens

ELISA described in section 3.2.1 was used to compare the concentrations of the Lewis antigens in the affinity purified dialysates and saliva samples.

Detection and quantification of H antigen in the dialysate was also carried out with an ELISA (Fig 4.1). The composition of the buffers used in the experiment are given in section 2.2. The wells of the polystyrene plates were coated overnight at 4^0C with 100 ul of PBS or 10-fold dilutions of the samples and control saliva in sodium carbonate buffer (50 mM, pH 9.6). All further procedures were carried out at room temperature. Washing buffer was used for washing throughout. After 3 washes, the wells were incubated with blocking buffer for 15 min. After 2 more washes, 100 ul

Figure 4.1. ELISA for detection of H antigen.



of biotin-labelled UEAI (Sigma, Poole, Dorset, UK) (5 ug/ml in blocking buffer) were added to each well for 30 min. After washing 3 times, 100 ul of streptavidin-biotinylated horseradish peroxidase complex (Amersham, Amersham, UK) diluted 1/100 in blocking buffer were added to the wells for 20 min. After 3 washes, 100 ul of activated substrate solution were added to each well. The reaction was terminated after 5-10 min by 50 ul of 12.5% H_2SO_4. The colour changes were measured on an ELISA plate reader (MR 700) (Dynatech).

4.2.4. Virus inhibition studies

One-day old freshly confluent layers of HEp-2 cells grown in 24-well tissue culture plates in 5% CO_2 in air were used in the assays. Infectious particles of RSV (Edinburgh strain) [Oglivie *et al.*, 1981] were incubated with different antigen preparations described below and adsorbed to cell monolayers for 60 min at 37^0C in 5% CO_2. The supernatant was removed and the culture plates incubated in overlay medium for 3-4 days until cytopathic effects (CPE) (syncytia and plaques) appeared in the monolayers. The monolayers were fixed and stained by crystal violet stain and examined for CPE.

4.2.4.1. Incubation of viruses with antigens

4.2.4.1.1. Affinity purified antigens

Virus suspensions containing 200 plaque forming units (p.f.u.) per 100 ul were incubated with an equal volume of purified blood group antigens or PBS as control at 37^0C for 60 min. Each sample was diluted with 600 ul of MM. The diluted virus suspensions were added in 200 ul volumes/well to 4 wells for each antigen and the viruses allowed to adsorb to cell monolayers. In some wells, MM or the antigen preparations (1/2 in MM) (200 ul/well) were added as controls for detection of any cytotoxic effects.

4.2.4.1.2. Synthetic glycoproteins

The glycoconjugates listed in Fig 4.1a consisted of blood group antigens purified from human milk conjugated with human serum albumin (Biocarb). The glycoconjugates were diluted in distilled water to 1mg/ml which contained 2-2.5X10^{-4} M of the glycoproteins. Virus suspensions (100 ul) containing 1000 p.f.u. of RSV were incubated with 20 ul of the glycoconjugate preparations under the same conditions used for antigens purified from saliva. The supensions were diluted with 1500 ul of MM and distributed to wells (200 ul/well) and the virus allowed to adsorb to cell monolayers as described above.

4.2.4.1.3. Saliva samples

Incubations of viruses with boiled saliva from secretors and non-secretors were also performed. Saliva samples (20 ml) were boiled for 20 min to inactivate antibodies, enzymes, bacteria and viruses and centrifuged at 1000 g for 10 min. The supernatant was diluted in an equal volume of MM. Virus suspensions containing 200 p.f.u. per 100 ul were incubated with equal volumes of diluted saliva or PBS control at 37^0C for 60 min. Each sample was diluted with 600 ul of maintenance medium (MM) and distributed (200ul/well) as described above.

4.2.4.1.4. UEAI

Doubling dilutions of UEAI ranging from 1/500 to 1/8000 in MM were incubated with the virus suspensions and the viruses were adsorbed to the cells as described above.

4.2.4.1.5. Synsorb beads with blood group specificities

In one experiment, viruses were incubated with Synsorb beads with Le^a, Le^b, H-type

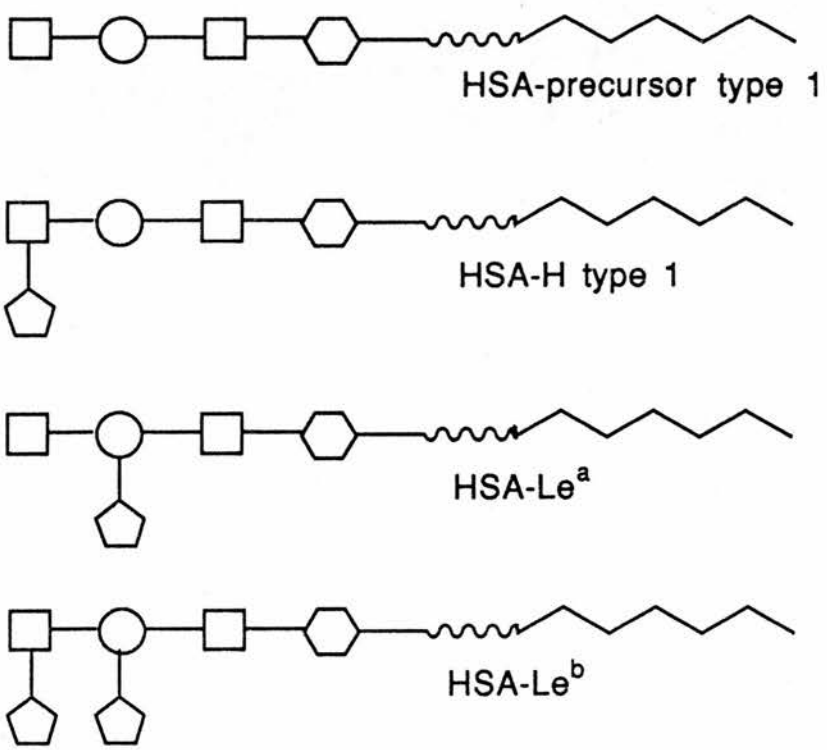


Figure 4.1a.Lewis and H blood group antigens purified from human milk and conjugated to human serum albumin (HSA) ⟋⟍⟋ through a spacer acetylphenylendediamine ∿∿ . Each mole of HSA contains 10-20 moles of oligosaccharide moieties. (Symbols as in Figure 1.1).

1 or H-type 2 determinants. The beads (30 mg) were suspended in absolute alcohol for 30 min. They were washed 3 times with PBS by centrifugation at 50 g for 5 min. Virus suspensions (200 p.f.u. in 250 ul) were incubated with the beads for 60 min at 37^0C with gentle shaking. The beads were allowed to settle. The supernatant from each sample was diluted with 600 ul MM and transferred to cell monolayers , 200 ul/well into 4 wells. The viruses were allowed to adsorb and the experiments carried out as described above.

4.3. Results

4.3.1. Detection of H and Lewis antigens on HEp-2 cells

4.3.1.1. Fluorescence microscopy

Le^a was not detected on cell monolayers. A few fluorescent specks were visible on a minor proportion of cells on monolayers treated with anti-Le^b and anti-H_2 antibodies. All cells treated with UEAI, however, were fluorescent.

4.3.1.2. Flow-cytometry

The data from flow cytometry with indirect and direct immunofluorescent techniques show anti-Le^b and anti-H_2 at all the dilutions bound to HEp-2 cells but anti-Le^a did not. Fluorescence recorded for HEp-2 cells treated with anti-Le^b and anti-H_2 at dilution 1/5 was comparable with that for BEC with these antibodies. Fluorescence of HEp-2 cells treated with anti-Le^a at this dilution was, however, much less compared with BEC (Fig 4.2). A higher proportion of fluorescent cells with higher means of fluorescence were detected with FITC-conjugated UEAI compared with anti-H^2.

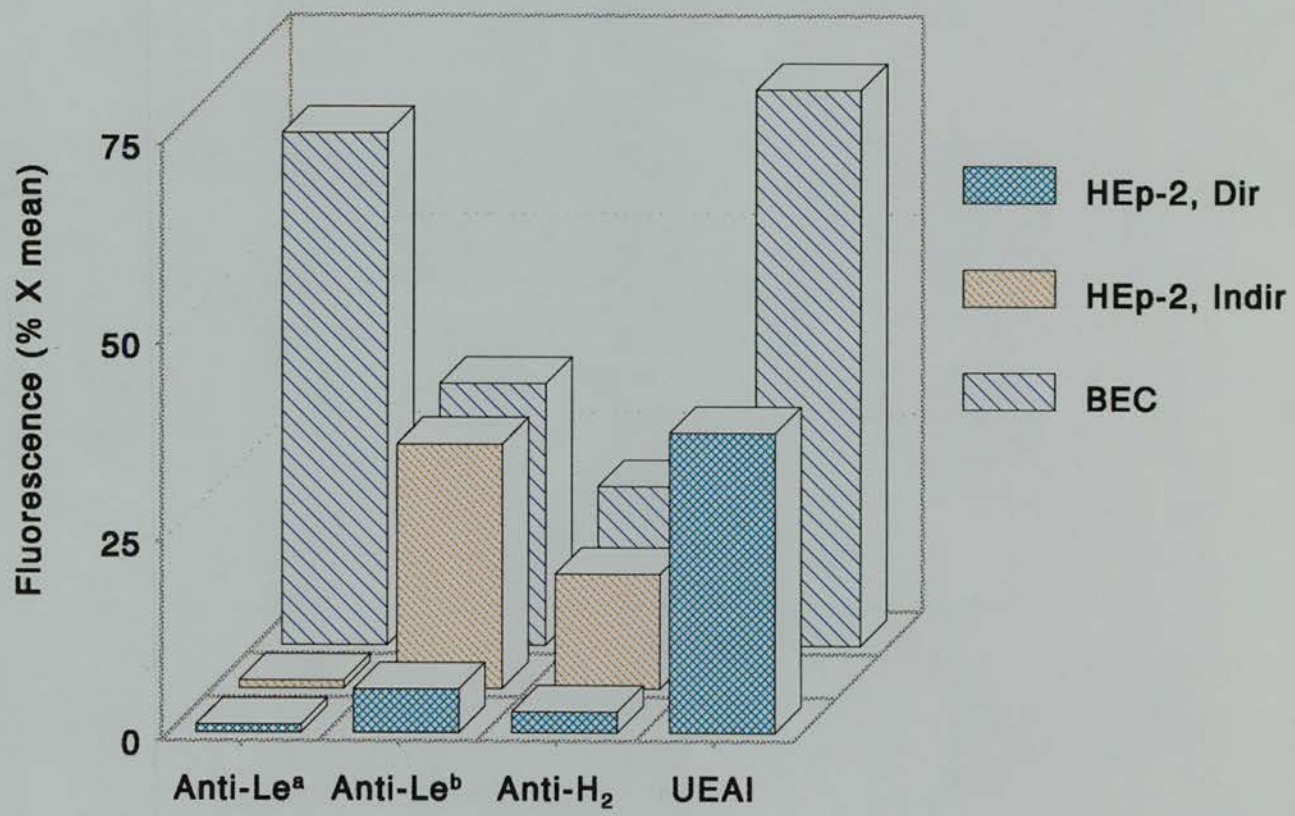


Figure 4.2. Comparison of binding of monoclonal antibodies to Lewis and H-type 2 antigens and FITC-conjugated UEAI to buccal epithelial cells (BEC) and HEp-2 cells: binding of antibodies were assessed by both indirect and direct fluorescent techniques

4.3.2. ELISA for Lewis and H antigens in affinity purified preparations

The amounts of Le^a, Le^b and H antigens detected by ELISA in the dialysate solutions of affinity purified antigens for virus inhibition assays were compared with those in corresponding saliva samples (Table 4.1). The supernatants from the Sepharose beads were also tested in the ELISA. These solutions contained high concentrations of antigens and were recycled with the beads to obtain more antigen.

4.3.3. Virus inhibition studies

4.3.3.1. Lewis antigens

Results of 5 assays suggest that RSV was not inhibited by affinity purified Le^a or Le^b antigens. In 3 more assays with glycoconjugates containing precursor chain or precursor chain with Lewis determinants, virus infectivity was not altered. Incubation of viruses with Synsorb beads linked with synthetic blood group determinants, Le^a, Le^b or H, caused about 50% decrease in p.f.u. in all cases compared with controls (Fig 4.3). The experiment was not repeated because the beads without any blood group antigens to be used as control were not available from the manufacturer.

4.3.3.2. Incubation of viruses with H-type 1 antigen

A preparation of purified H-type 1 antigen was obtained from saliva from a Lewis negative donor but was sufficient for one experiment only. The solution was used in doubling dilutions in an inhibition assay. Incubation of RSV with the preparation decreased the number of plaques or syncytia by more than 90% (1st. experiment in Fig 4.4). A dose related inhibition in virus infectivity was also observed in this experiment. The inhibitory effect of H antigen was, however, not observed with

Optical density measurements

Antigen	Purified sample	Saliva sample
Le^a	0.680	0.455
Le^b	0.212	0.375
H	0.178	0.458

Table 4.1. Amount of Le^a, Le^b or H_1 in saliva or affinity purified preparations detected by ELISA. Saliva samples used for comparison of Le^a were from non-secretors and those for Le^b and H_1 were from secretors.

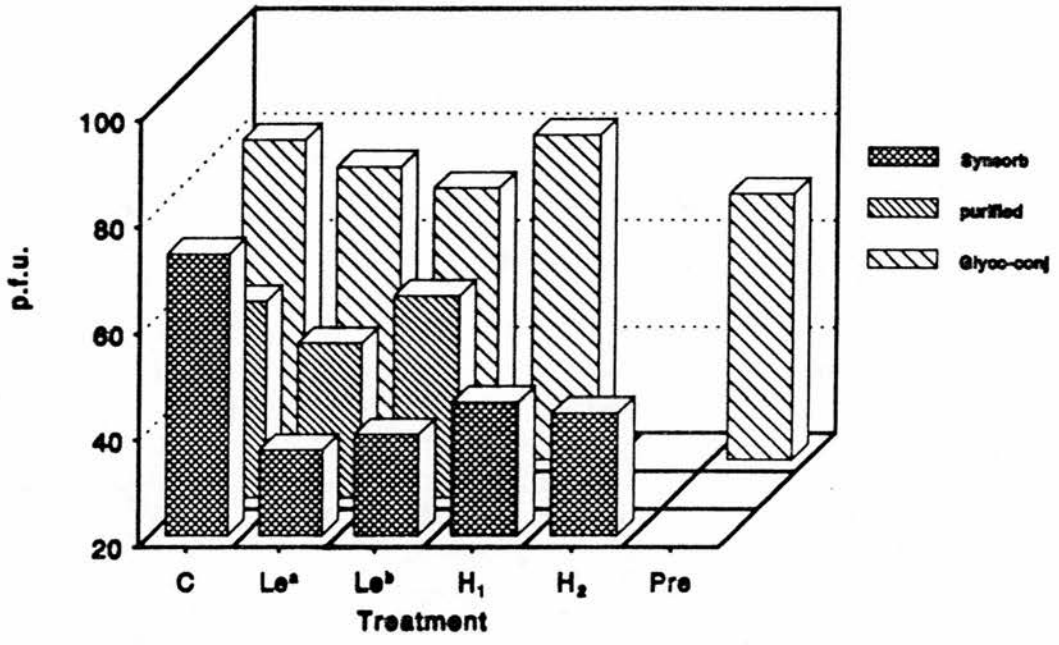


Figure 4.3. Effect of incubation of RSV with affinity purified Lewis or H antigens or glyco-conjugates or Synsorb beads with blood group activity on plaque formation. (Pre-precursor chain, C-virus control)

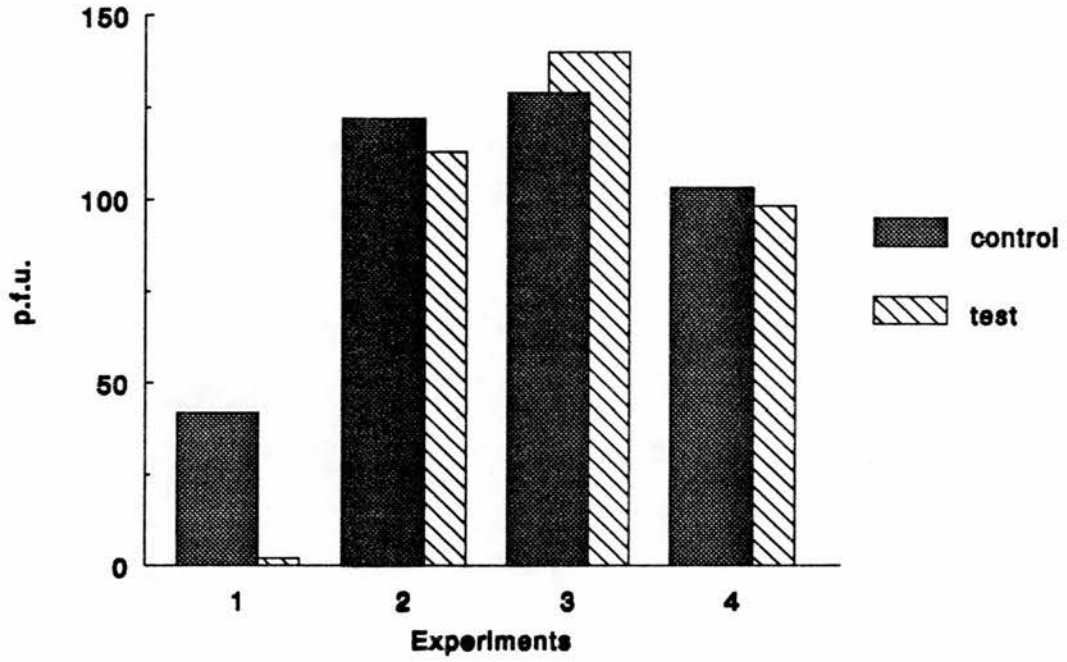


Figure 4.4. Effect of incubation of RSV with affinity-purified H antigen on plaque formation.

preparations purified from saliva from the same donor on 3 later occasions (Fig 4.4). Viruses were also incubated with diluted saliva samples from the donor in one experiment but no effect on the infectivity was observed.

The inhibitory effects observed in the first experiment might be due to contamination of the antigen solutions with sodium azide, a constituent of NET buffer or with molecules of UEAI detached from Sepharose beads during the process of purification of H antigen. To exclude the latter possibility, the virus suspensions were incubated with various dilutions of UEAI. UEAI was not toxic to the cells in dilutions ranging from 1/400 to 1/8000 of the solution used in the purification assays. UEAI did not cause inhibition of RSV infection of the cells.

4.3.3.3. Incubation of viruses with saliva

Treatment of viruses with boiled saliva from Lewis positive secretors or non-secretors did not significantly affect the infectivity in 4 experiments (Fig 4.5).

4.3.3.4. Incubation of HEp-2 cells with Lewis and H antigens

The virus particles could not be washed after incubation with the antigens. Controls were added in each experiment to assess the effects of antigens on cell monolayers and on the infectivity of RSV. The antigen preparations were not toxic to the cells. Treatment of monolayers with the preparations did not greatly affect the RSV-infectivity (Fig 4.6).

4.4. Discussion

Fluorescence was detected more accurately with flow cytometry compared with microscopy due to the difference in sensitivity between the two methods. Binding of monoclonal anti-Le^b and anti-H_2, but not anti-Le^a to the cells was detected. Higher

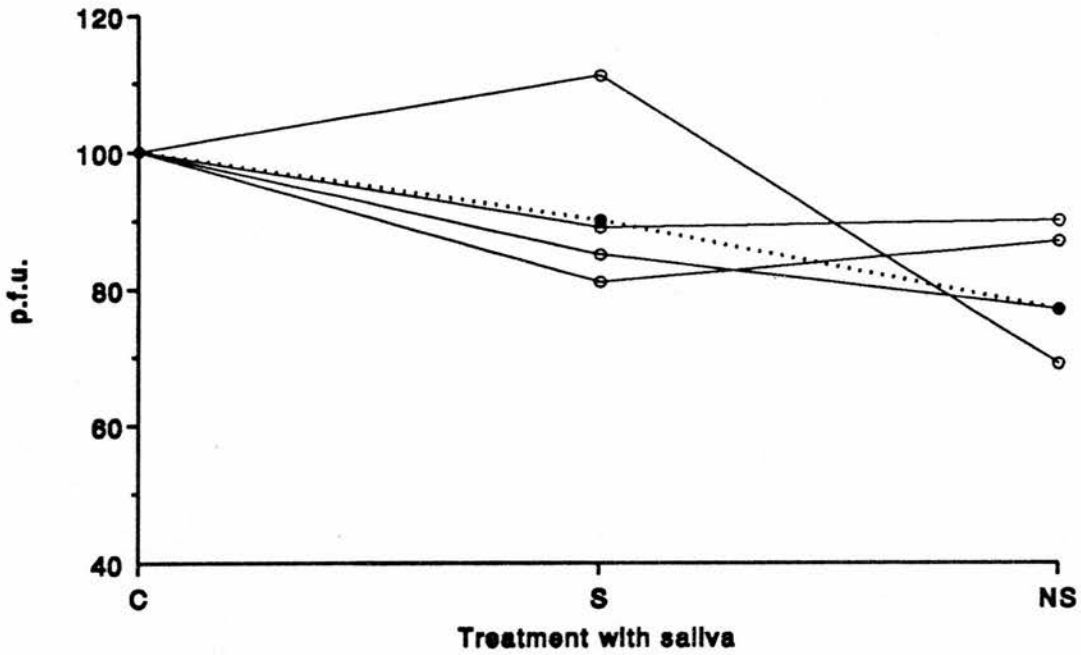


Figure 4.4. Effect of incubation of RSV with saliva samples from secretors (S) and non-secretors (NS) on plaque formation. (The broken line in the graph indicates average readings of the 4 experiments.)

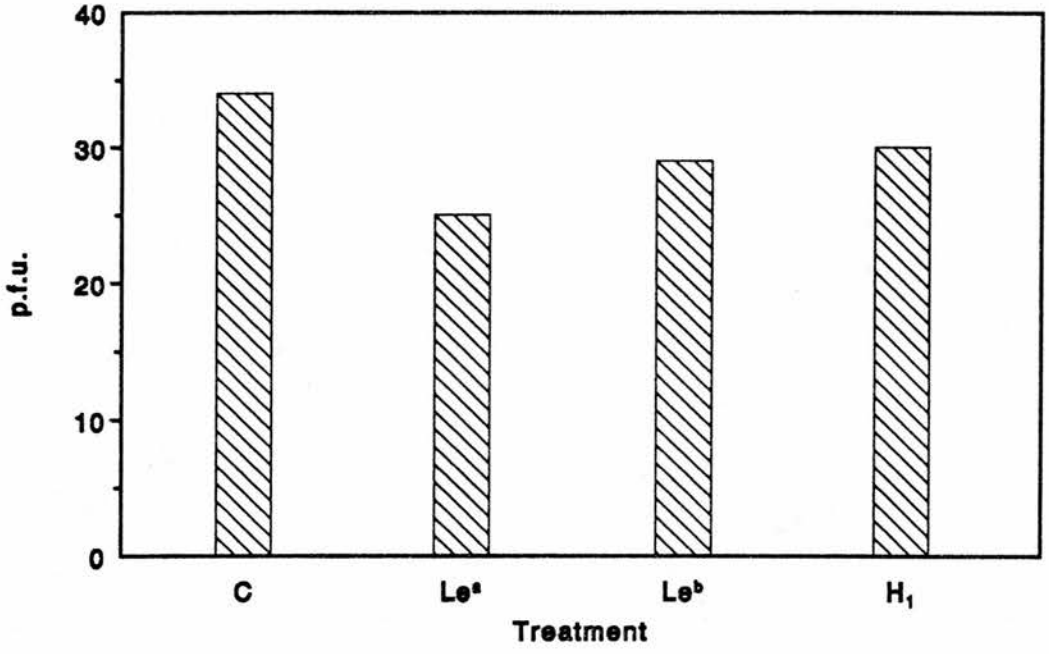


Figure 4.6. Effect of incubation of HEp-2 cell monolayers with affinity purified Lewis and H antigens on plaque formation by RSV.

proportions of fluorescent cells with higher mean fluorescence were detected with UEAI compared with anti-H_2 antibodies in this study. UEAI detects fucose on both H-type 1 and H-type 2 and other fucose containing molecules.

The use of plaque assays to study cellular receptors for RSV is limited by a number of factors. A lysate of RSV-infected HEp-2 cells was used as a source of virus particles in these experiments. Blood group antigens tested for their inhibitory effect on virus infectivity were presumably already present in the lysate. RSV cannot be easily isolated and purified from the lysate because 90% of virus particles remain associated with the cell membrane [Levine and Hamilton, 1969]. These studies cannot be conclusive since blood group antigens with the adherent viruses on glycoproteins might be adsorbed onto the cell surface enhancing infectivity.

The results suggest that affinity purified blood group substances with Lewis or H blood group activity did not decrease the infectivity of RSV. Lewis and H blood group antigens bound to HSA added in a proportion of approximately 10^{14} blood group determinants per infectious particle of RSV did not reduce the virus infectivity for HEp-2 cells. The inhibitory effect of a preparation of purified H-type 1 antigens observed for the first experiment could not be explained but did not appear to be due to contamination of the preparation with UEAI molecules. The effect was, however, not reproducible with different preparations of the antigen purified from saliva from the same donor.

The results from experiments involving incubation of viruses with Synsorb beads support this conclusion. Beads with H and Lewis determinants uniformly reduced the number of infective particles, suggesting that the effect was non-specific. This could most likely be due to physical damage to the virus particles by the beads or due to trapping of virus particles that are associated with debris of cell membrane in the bead-bed.

Assessment of binding of purified blood group determinants with purified G and F glycoproteins might be a more precise way to analyse the proposed interaction of blood group antigens with RSV surface antigens.

Chapter 5

Binding of *Neisseria meningitidis, Haemophilus influenzae* and *Staphylococcus aureus* to RSV-infected cells

5.1. Introduction

There is extensive evidence that virus infections are prediposing factors for invasive bacterial disease or carriage of potentially pathogenic bacteria (1.4.8). Most information has come from studies of influenza virus and bacteria causing pneumonia such as pneumococci and staphylococci. Infection due to respiratory syncytial virus (RSV) and meningitis due to *N. meningitidis* and *H. influenzae* occur in the same age groups and during similar periods of the year [Greenwood, 1984; Kim *et al.*, 1973; Noah, 1989; Jones and Kaczmarski, 1991; Nazareth *et al.*, 1992] (Fig 1.2). In the present study, the effect of viral infection on the initial binding of bacteria to epithelial cells was investigated.

The HEp-2 cell line was used in assays to determine the effects of RSV infection on bacterial binding. The HEp-2 cell line is derived from a human laryngeal carcinoma and consists of non-ciliated epithelial cells. The cells are permissive to RSV and have also been used for bacterial attachment studies [Stephens, 1989; Layh-Schmitt *et al.*, 1989]. As the model on which the experiments were based was that of staphylococcal binding to virus infected cells, a strain of *Staph. aureus* was also included in these studies. The bacterial binding was analysed by flow cytometry (2.7).

In the present study, the hypothesis tested was that HEp-2 cells infected with RSV might bind meningococci, *H. influenzae* type b (Hib) and *Staph, aureus* to a greater

extent than uninfected cells.

5.2. Materials and Methods

5.2.1. Cells and Virus

HEp-2 cells (Flow Laboratories) between passages 400-420 were used for the attachment assays. RSV (Edinburgh strain) (a subgroup A strain) [Ogilvie *et al.*, 1981] was used at the 5th to 7th passages to infect the HEp-2 cells.

5.2.2. Bacteria

A variety of strains of meningococci, Hib (Table 2.1 and 2.2) and a non-toxigenic (pyrogenic toxins) strain of *Staph. aureus* (8532) (defined by National Collection of Type Cultures, NCTC) were used in these assays. Bacteria were cultured as described in section 2.6. Briefly, meningococci were grown on MNYC medium and for some experiments on BBA or GC medium while Hib were grown on BBA for all the experiments. *Staph. aureus* were cultured on blood agar.

5.2.3. Binding of meningococci to HEp-2 cells in monolayers

HEp-2 cells ($7.5x10^4$/ml in GM) were seeded onto glass coverslips (1 ml/well) in 24 well culture plates [Costar] and incubated in 5% CO_2 in air overnight at 37^0C. The subconfluent monolayers were infected with RSV at different M.O.I. ranging from 0.001 to 1.5 p.f.u./cell. Virus was added in 200 ul of maintenance medium (MM) to each well for one hour at 37^0C. Following adsorption of the virus, the inoculum was discarded and the infected cells were incubated in MM for 24 hours in 5% CO_2 at 37^0C before they were used in attachment studies with the bacteria.

The bacteria were harvested, washed three times in DPBS by centrifugation at 2500 g for 15 minutes and resuspended in MM without antibiotics by vigorous pipetting to

disperse clumps. The bacterial concentration was determined by the optical density method (section 2.6.2) and adjusted to provide a range of ratios of bacteria per cell. The cell monolayers were washed and incubated at 37^0C with 200 ul of the bacterial suspension for various intervals. The coverslips were washed three times and fixed in absolute ethyl alcohol for five minutes at room temperature. The coverslips were stained by Gram's stain and examined by light microscopy with an oil immersion lens. The number of cells counted, number of cells binding bacteria and number of bacteria per positive cell were recorded. More than 100 cells were counted for each coverslip. A binding index for each coverslip was obtained by multiplying the proportion of cells which bound bacteria by mean numbers of bacteria counted.

5.2.4. Binding of meningococci to HEp-2 cells in suspension

Overnight monolayer cultures of HEp-2 cells in culture flasks (Costar) (25 cm^2) were infected with RSV at different M.O.I. ranging from 0.001 to 1.0. Viruses (1 ml, infected cell lysate) were adsorbed for 1 hour and replaced with 10 ml MM. The cultures were incubated overnight at 37^0C.

In one experiment, the cell monolayers were also incubated with inactivated viruses as above to assess the binding of strain C:2b:P1.2 on mock-infected HEp-2 cells. The virus suspension (5ml) ($1x10^6$ p.f.u./ml) in a petri dish was exposed to 192 mJ/cm^2 of ultraviolet (UV) light. The suspension was tested for inactivation of viruses by a plaque assay. Cell monolayers in 25 cm^2 flasks were incubated with 1 ml/flask of UV-treated suspension. Cultures of mock-treated monolayers were incubated overnight as above.

Cultures were rinsed twice with DPBS, and ethylenediamine tetra acetic acid (EDTA) (0.05%) was applied, 1 ml per each flask at 37^0C for 5-10 minutes to detach the cells from the surface. MM (5 ml) without antibiotics was then added to the cells to

terminate EDTA-activity. After centrifugation at 460 g for 7 minutes, the cells were resuspended in MM without antibiotics, counted and adjusted to $1x10^6$/ml. The cell suspensions were gently rotated at 37^0C while the bacteria were prepared. The time from the end of adsorption of viruses to monolayers to the addition of bacteria was 24 hours (± 1hr).

The bacterial suspensions were labelled with fluorescein isothiocyanate (FITC) (Sigma) or rhodamine (Eastman Organic Chemicals) as described in section 2.6.4. The labelled bacteria were washed three times with DPBS and resuspended in MM without antibiotics. Suspensions of pilate strain were incubated for 2 hours at 37^0C in 5% CO_2 to allow regeneration of bacterial pili.

The concentration of the suspension was determined by the optical density method (2.6.3) and adjusted to provide a range of ratios of bacteria per cell. The bacterial suspensions (200 ul) were incubated with equal volumes of suspension of HEp-2 cells for different periods at 37^0 C with gentle rotation at 60 rpm in an orbital incubator (Gallenkamp). At the end of each incubation period, the cells were washed three times by centrifugation at 460 g for 7 min. The cells were resuspended in 200 ul of DPBS and fixed with 100 ul of 1% buffered paraformaldehyde (Sigma) [Lanier and Warner, 1981]. The samples were kept in the dark at 4^0C until analysed within 3 days of the assay, mostly on the second day after the experiment.

Each sample was analysed by flow cytometry with an EPICS-C [Coulter Electronic, Luton, UK]. The results were expressed as binding indices (BIs) calculated for each sample by multiplying the percentage of fluorescent cells obtained from 'Immunoanalysis' by the mean fluorescence.

The cells were tested for the proportion of RSV-infected cells in each sample using immunofluorescent methods described in section 2.5.3.

5.2.5. Binding of *H. influenzae* and *Staph. aureus* to HEp-2 cells

The same method was used to investigate the binding of Hib and staphylococci to HEp-2 cells infected with RSV at M.O.I. 1.0. Hib were grown on boiled-blood agar medium for 48 hours. FITC-labelled bacteria were incubated with cells in ratios of 50, 200 and 800 bacteria per cell for 2 hours [St. Geme and Folkow, 1990]. Staphylococci were grown on blood agar for 24 hours. The cells were incubated with FITC-labelled bacteria using 2 ratios of bacteria:cell, 320 and 640, for 30 min at 37^0C [Blackwell *et al.*, in press]. After washing, the cells were analysed in the flow cytometer as described above.

5.2.6. Statistical methods

The results were analyzed with the statistical package 'Minitab' using multiple regression on the logarithms of the BIs of the samples. For meningococci, dummy variables were included to adjust for daily variations in the experiments and different ratios of viruses and bacteria per cell. The coefficients from this analysis were used to estimate binding relative to that found for no virus and 10 bacteria per cell by taking antilogarithms. Examination of the distribution of residuals from the fitted models suggested that the logarithmic transformation had given an acceptable fit to a normal distribution. A sign test was used to test for consistency of excess binding of Hib to RSV-infected HEp-2 cells. A paired t-test was applied to the data from assays with staphylococci

5.3. Results

5.3.1. Detection of RSV infected cells

The percentage of RSV-infected cells for each M.O.I. was determined by two

methods, fluorescence microscopy and flow cytometry. The values obtained by the two methods were comparable, and the proportions of infected cells in the samples ranged from <10% to >80% at the time of the attachment assay (Table 5.1). No cytopathic effect was noted at this stage. Random sample of RSV-infected cells did not show significant change in fluorescence when analysed by flow cytometry on the second, third or fourth day of the experiment.

5.3.2. Attachment of meningococci to cell monolayers

5.3.2.1. Determination of optimum incubation period with monolayers

Binding of meningococci at ratios ranging from 40 to 320 bacteria per cell with the uninfected monolayers of HEp-2 cells was examined for different incubation periods (Fig 5.1).

An incubation period of 30 min was used for the following experiments with meningococci. At this incubation period the binding was at mid-range levels and cells were not affected. Longer incubation with the bacteria (>1 hour) caused retraction, rounding and toxic effects on the cells (Fig 5.1.a).

5.3.2.2. Bacterial binding to RSV-infected and uninfected HEp-2 cells in monolayers: assessed by microscopy

Binding of meningococci to the uninfected and RSV-infected HEp-2 cells at various ratios of bacteria per cell was examined (Fig 5.2). At ratios of 250-500 bacteria/cell more bacteria bound to cells in monolayers infected with RSV at M.O.I. 1.5 compared with uninfected cells. At lower M.O.I. the results were equivocal. Counting by microscopy was, however, subject to errors. Two counts made on some monolayers gave significant variations (Fig 5.3). As a result the method was

Table 5.1. Proportion of virus-infected cells in samples of HEp-2 cells infected with different multiplicity of infection (M.O.I.) of RSV.

M.O.I.	%
0.001	<10
0.010	20-30
0.10	40-50
1.0	>80

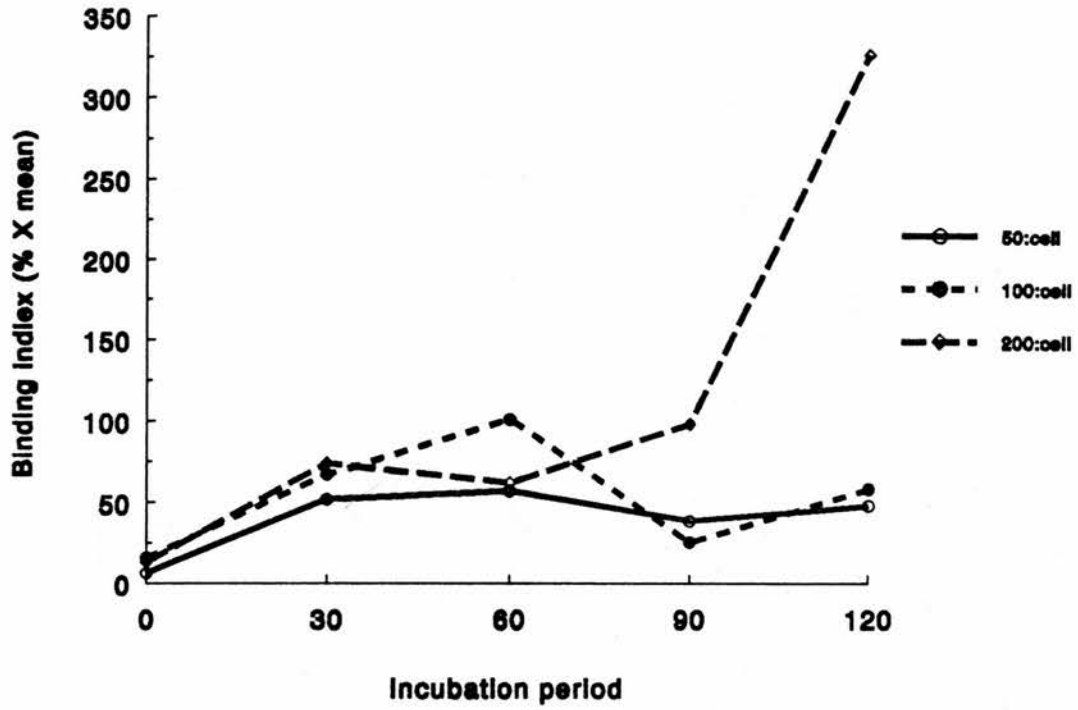


Figure 5.1. Binding of strain C:2b:P1.2 to HEp-2 cells after different incubation periods determined by microscopy.

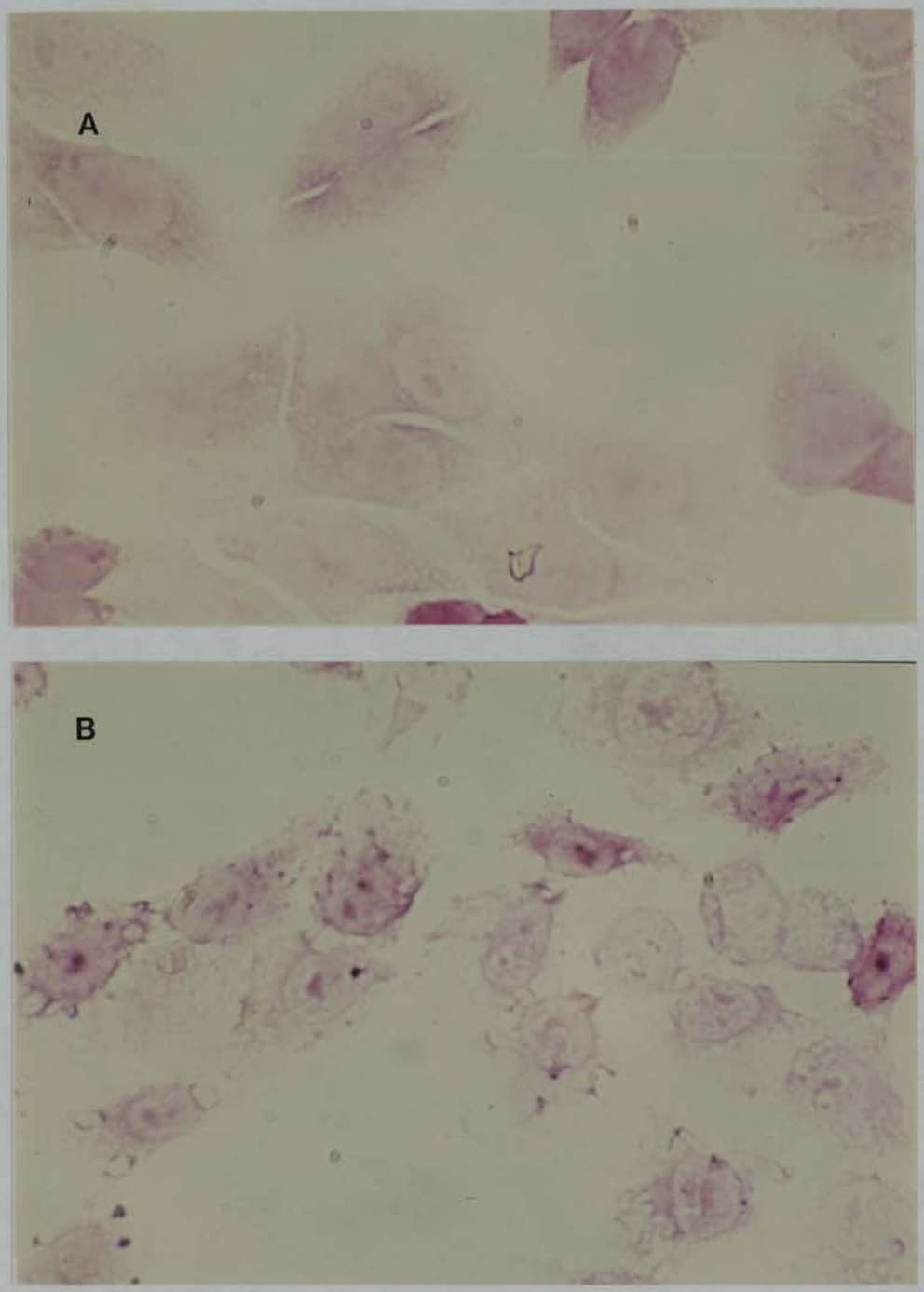


Figure 5.1.a. Replicate monolayers of HEp-2 cells incubated with 250 bacteria (C:2b:P1.2) per cell for 30 min (A) and for 120 min (B). (magnification: X1600)

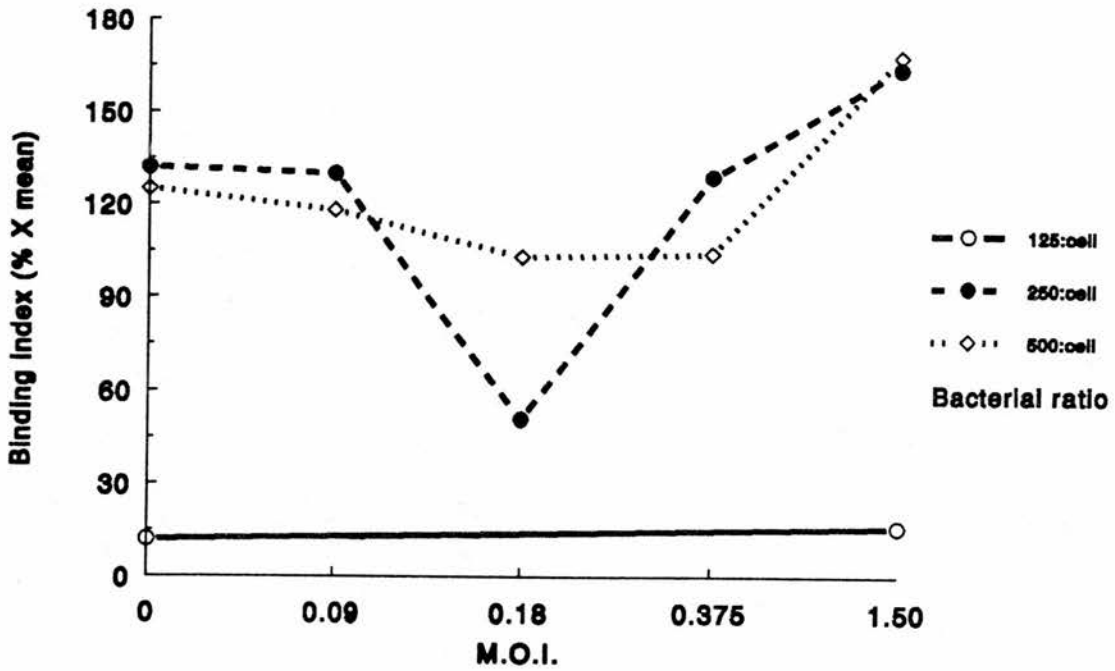


Figure 5.2. Effect of multiplicity of infection (M.O.I.) of RSV On binding of strain C:2b:P1.2 to HEp-2 cell monolayers.

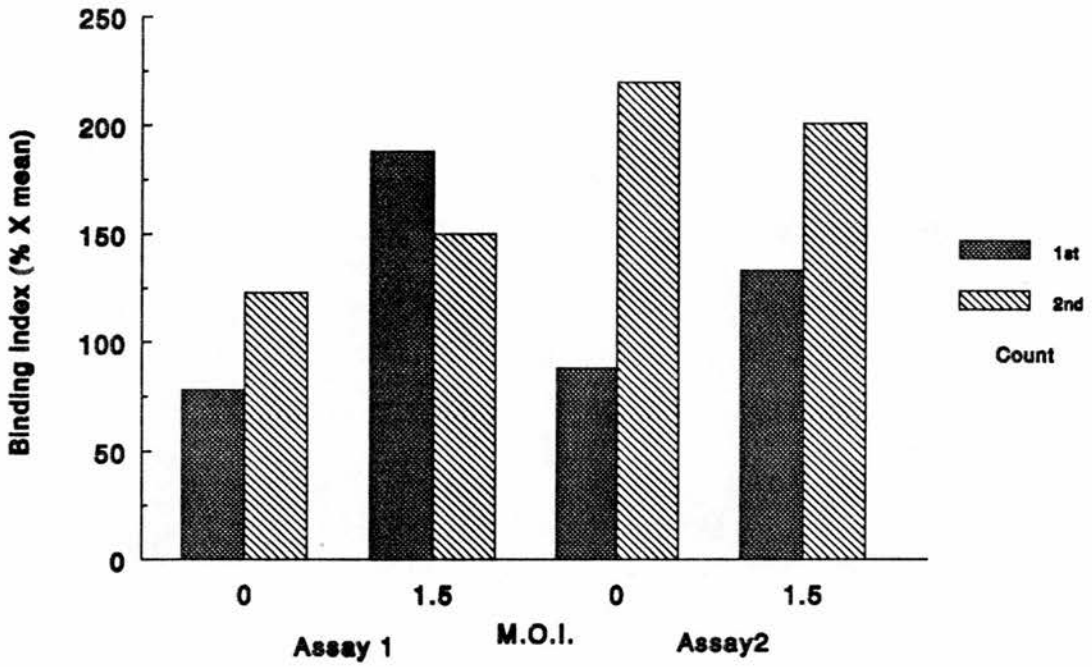


Figure 5.3. Difference between two counts of the same monolayers of uninfected and RSV-infected HEp-2 cells determined by microscopy.

abandoned.

5.3.3. Attachment of meningococci to cells in suspension assessed by flow cytometry

5.3.3.1. Labelling of bacteria

In one experiment, meningococci were labelled separately with the fluorescent dyes, rhodamine [Darzynkiewicz *et al.*, 1982] or FITC, to examine their relative brightness and stability (Fig 5.4). Labelling with FITC was brighter than with rhodamine. Samples with rhodamine also became non-fluorescent within a week of labelling while FITC-labelled bacteria were more stable under identical conditions. There were no significant changes in fluorescence of FITC-labelled bacteria within 3 days of the experiment. For these reasons FITC was chosen to label the bacteria for the following experiments. Rhodamine-labelled bacteria were used in one experiment at ratios of 40, 80, 160, 320 and 640 bacteria:cell. Similar to experiments with FITC-labelled bacteria, HEp-2 cells infected with RSV at M.O.I. 1.0 bound more bacteria (C:2b:1.2) compared with uninfected HEp-2 cells.

5.3.3.2. Determination of optimum incubation period for attachment of meningococci to HEp-2 cells in suspension

The FITC-labelled bacteria were incubated with HEp-2 cells in suspension for various intervals. As with the experiments with monolayers, an incubation period of 30 min was found appropriate for the cells in suspension on the basis of adequate binding (Fig 5.5) and minimum debris visible on the two-parameter histogram of the flow cytometer.

5.3.3.3. Effect of media on binding

Initial studies were carried out with bacteria grown on NYC medium. The effect of

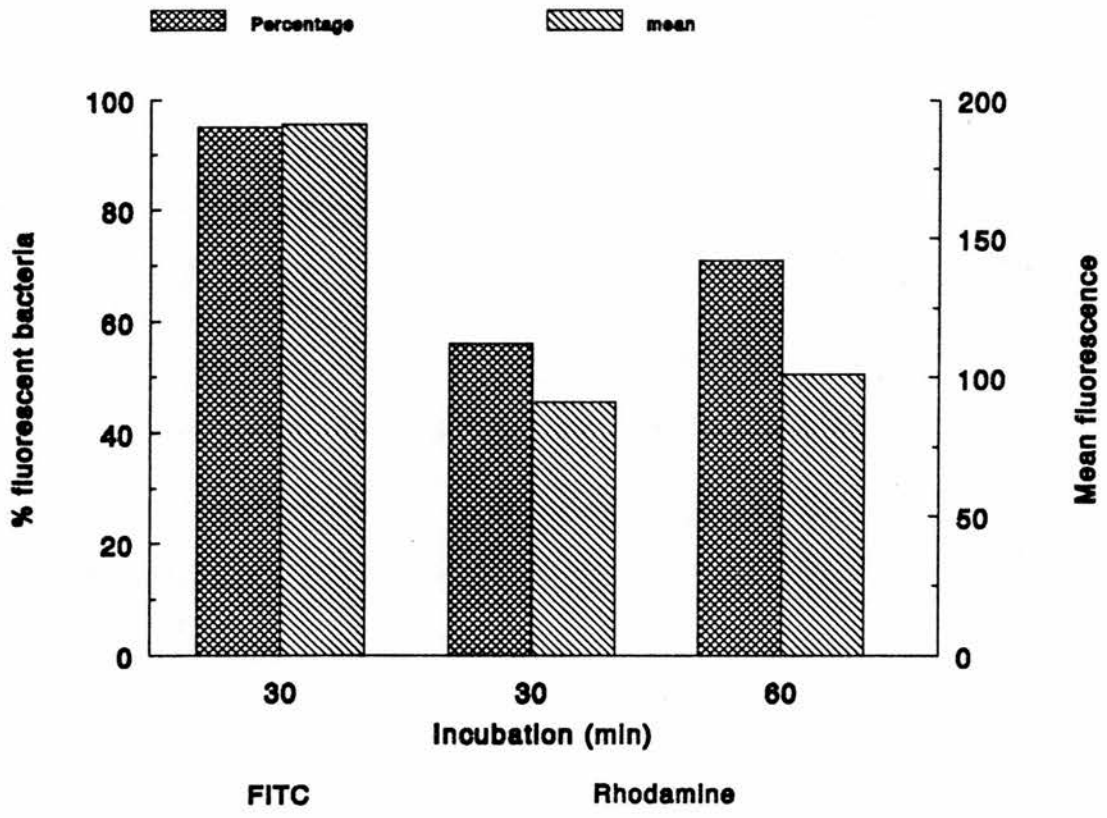


Figure 5.4. Fluorescence recorded for meningococci labelled with fluorescein isothiocyanate (FITC) or rhodamine.

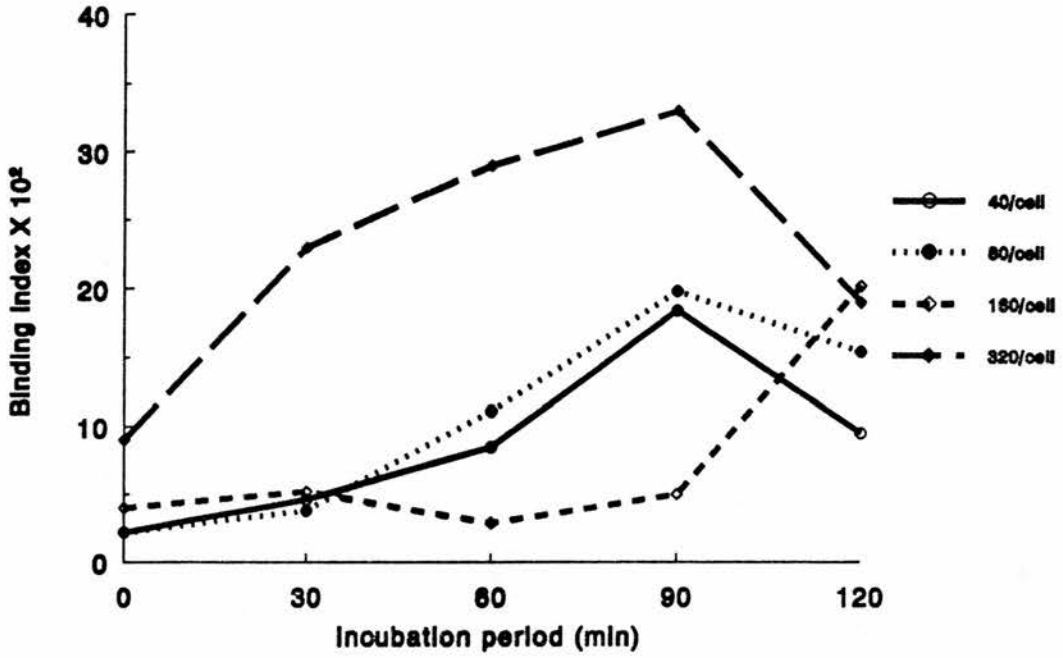


Figure 5.5. Binding of strain C:2b:P1.2 to HEp-2 cells after different incubation periods determined by flow cytometry.

RSV infection on binding of meningococci C:2b:P1.2 to HEp-2 cells is illustrated in Fig 5.6 and 5.7. The left hand peaks correspond to fluorescence recorded with uninfected cells following incubation with 320 bacteria per cell. The right hand peaks represent fluorescence observed with the cells infected with M.O.I. of 0.1 (fig 5.6) or 1.0 (Fig 5.7) following incubation with 320 bacteria/cell.

Table 5.2 and Figure 5.8 summarize the results obtained by flow cytometry assays to assess the effect of viral infection at various M.O.Is. on binding of bacteria. The results for 11 assays are expressed as estimated percentages of the binding index (BI) obtained with 10 bacteria per uninfected cell. The results indicate that prior infection of the cells with RSV at M.O.I. of 0.1 and 1.0 enhanced the binding of *N. meningitidis* at ratios of >80 bacteria per cell as illustrated in Fig 5.9 ($F(2.51)=37.37$, $P<0.001$).

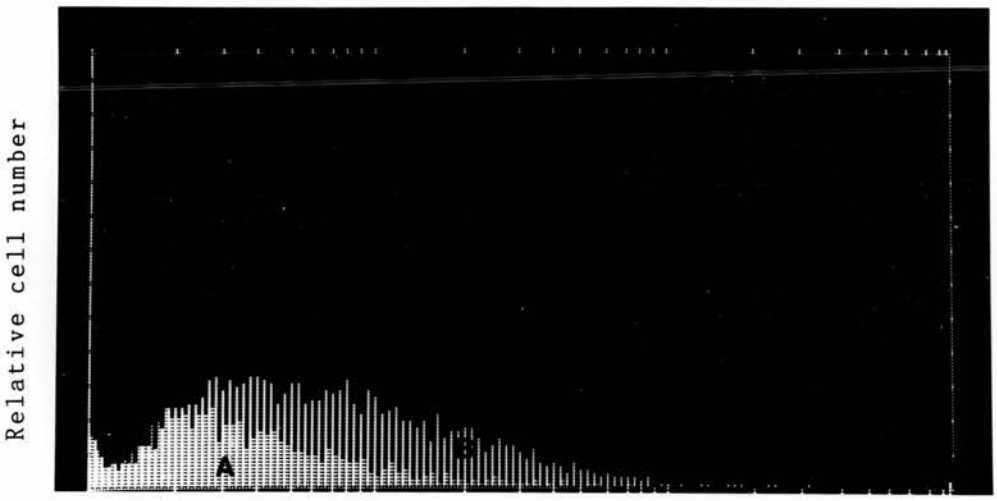
At lower ratios of bacteria per cell (10-20), the presence of viruses at any level did not change the binding level significantly; however, the cells infected with an M.O.I. of 0.001 demonstrated decreased bacterial binding at all ratios compared with uninfected cells; ($F(1.45) = 5.48$, $P<0.05$) (Fig 5.10).

Similar results were obtained with strain C:2b:P1.2 grown on BBA or GC medium incubated with HEp-2 cells infected with RSV at M.O.I. 1.0 at ratios of 40, 160 and 640 bacteria:cell (Fig 5.11).

5.3.3.4. Effect of mock-infection of HEp-2 cells on binding of bacteria

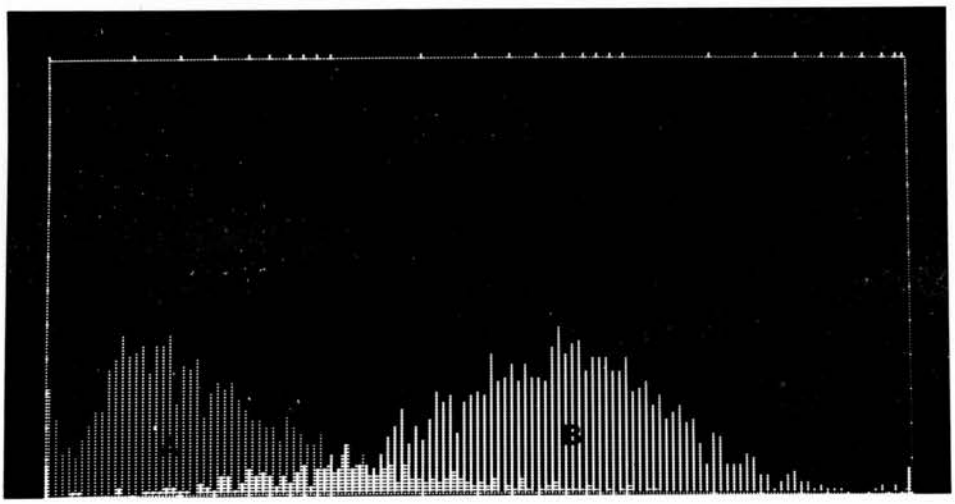
The results from the assay using UV-treated viruses show that the incubation of cell monolayers with suspensions containing one inactivated virus particle/cell did not significantly alter the binding of bacteria at different ratios of bacteria:cell compared with uninfected cells (Fig 5.12).

Figure 5.6



Binding of FITC labelled N. meningitidis to HEp-2 cells infected with RSV (MOI 0.1) (B) and uninfected cells (A).

Figure 5.7



inding of FITC labelled N. meningitidis to HEp-2 cells nfected with RSV (MOI 1.0) (B) and uninfected cells (A).

Estimated binding indices from multiple regression

No. of bacteria added per cell	Amount of virus added (M.O.I.) 0	0.001	0.01	0.1	1.0
10*	100	60	69	125	-
20	79	89	123	102	117
40	194	123	125	239	213
80	316	190	323	331	407
160	575	380	467	776	1288
320	1000	588	1148	1905	3311
640	1318	-	-	2818	5248
1000	2238	-	-	5623	11220

Table 5.2. Effects of infection of HEp-2 cells with RSV on binding of strain C:2b:P1.2. (* mean for M.O.I. = 0 and 10 bacteria per cell taken as 100.)

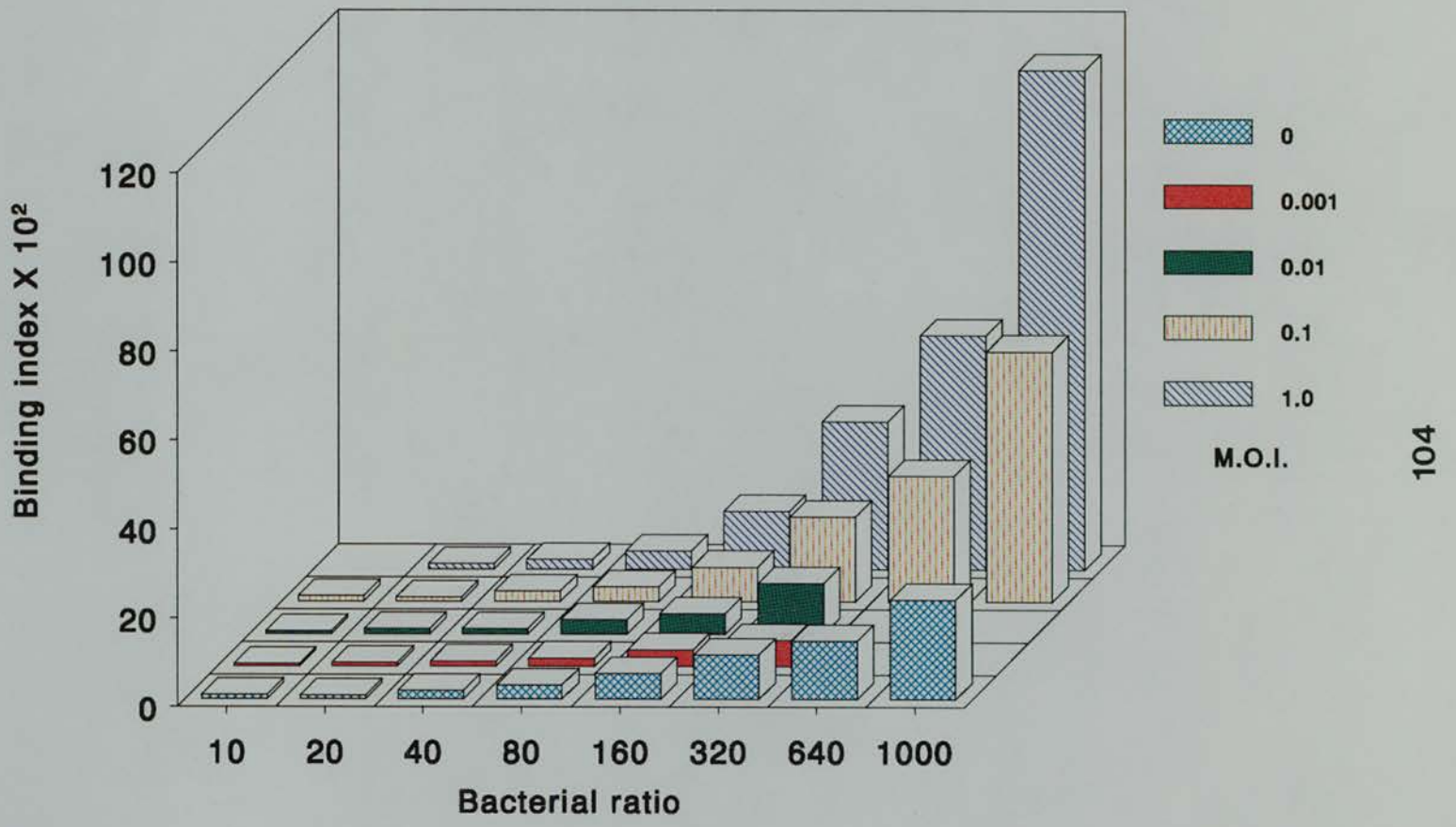


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Figure 5.8. Binding of strain C:2b:P1.2 to HEp-2 cells and HEp-2 cells infected with RSV at different M.O.I. at a range of ratios of bacteria per cell.

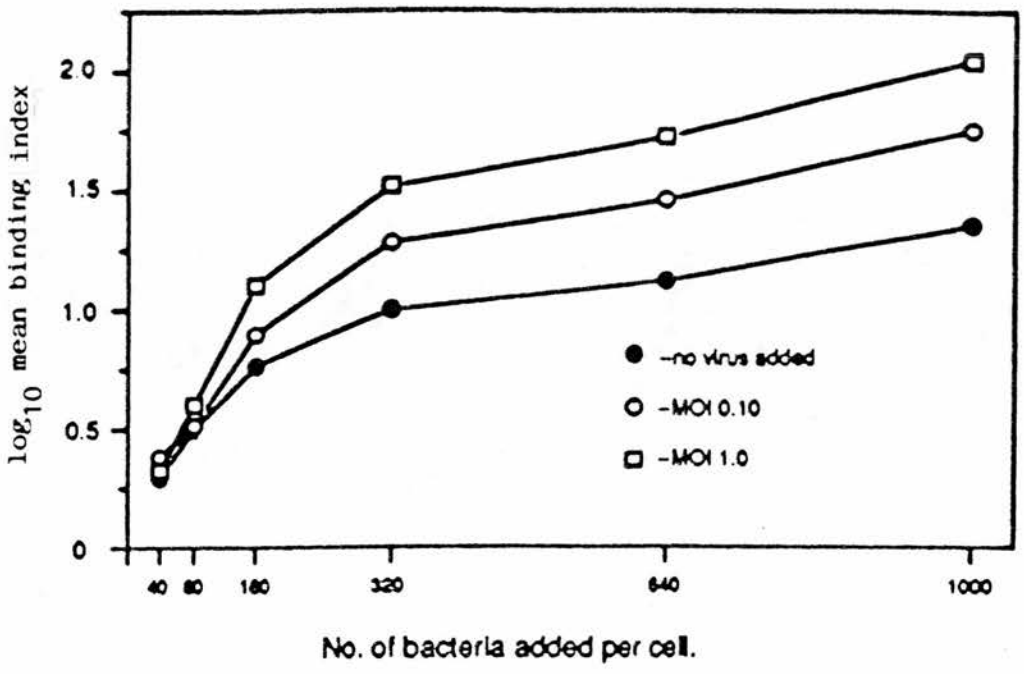

Fig 5.9 Effect of infection of HEp-2 cells with RSV at MOI of 0.1 and 1.0 on binding of Neisseria meningitidis to the cells.

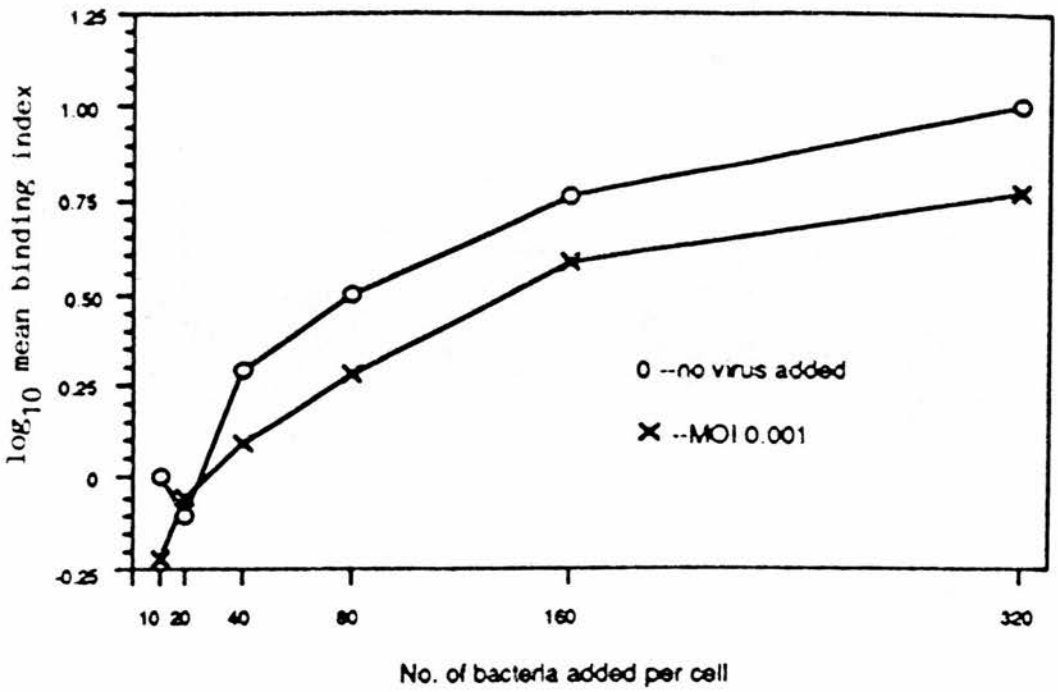

Fig 5.10 Effect of infection of HEp-2 cells with RSV at MOI of 0.001 on binding of Neisseria meningitidis to the cells.

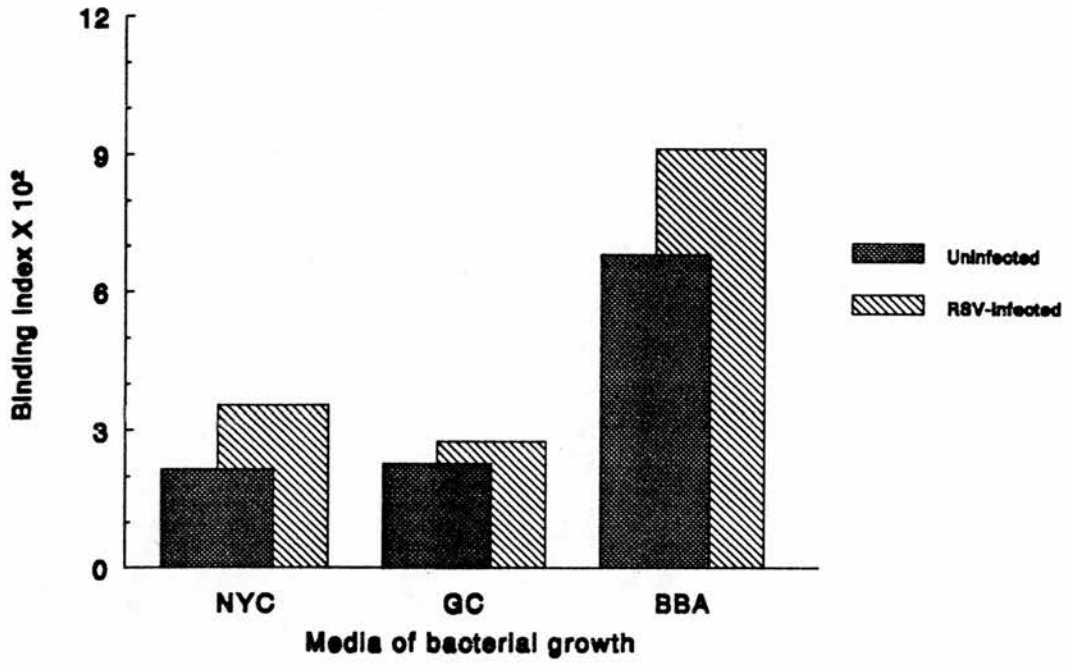

Figure 5.11. Cumulative binding of strain C:2b:P1.2 grown on NYC, GC or BBA at 3 ratios of bacteria: cell (text) to HEp-2 cells or HEp-2 cells infected with RSV at M.O.I. 1.0.

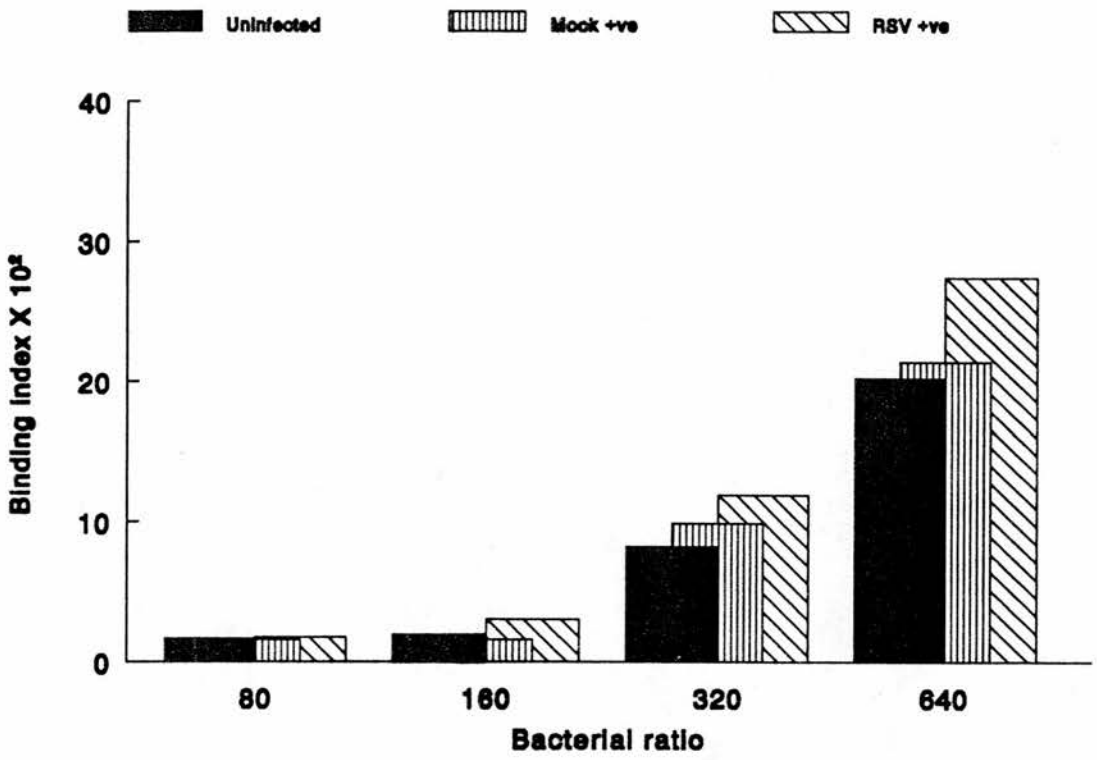

Figure 5.12. Effect of mock-infection of HEp-2 cells on binding of strain C:2b:P1.2.

5.3.3.5. Binding of other strains of *N. meningitidis*

Results similar to those found with strain C:2b:P1.2 for bacterial ratios 40, 80, 160, 320 and 640 bacteria:cell were obtained with the other strains of meningococci which expressed different serogroup, serotype and subtype surface antigens. Presence of pili on the strain Y:14:P1.2 did not affect the binding pattern (Fig 5.13).

5.3.4. Attachment of *H. influenzae* to HEp-2 cells in suspension

HEp-2 cells infected with RSV at M.O.I. 1.0 bound significantly more Hib at ratios 50, 200 and 800 bacteria:cell than uninfected cells. Although the effect varied significantly between experiments, the mean of log differences was positive in all 10 experiments using strain 14 ($P<0.01$, sign test). Median estimate of binding to RSV-infected cells as percentage of binding to uninfected cells was 127% (range 104% to 303%). RSV-infection of the cells enhanced bacterial binding at all ratios of bacteria tested. Although 5 strains of Hib (Table 2.2) differed in antigenic characteristics, they did not differ in the pattern of increased binding to RSV-infected cells compared with uninfected cells in 7 repeated assays, nor did the bacteria:cell ratio affect the binding significantly.

5.3.5. Attachment of *Staph. aureus* to HEp-2 cells in suspension

Effect of RSV-infection of HEp-2 cells at M.O.I. 1.0 on binding of *Staph. aureus* was significant in both ratios of bacteria:cell 320 and 640 (paired t-test); at the ratio 320:cell, $t=6.56$ ($P<0.001$) and at 640/cell, $t=4.13$ ($P<0.01$). The confidence limits (95%) for increase in binding due to RSV-infection as % of binding to uninfected cells were 112, 149 and 114, 161 percent respectively. No significant differences between assays with different ratios of bacterial:cell were noted ($t=0.12$).

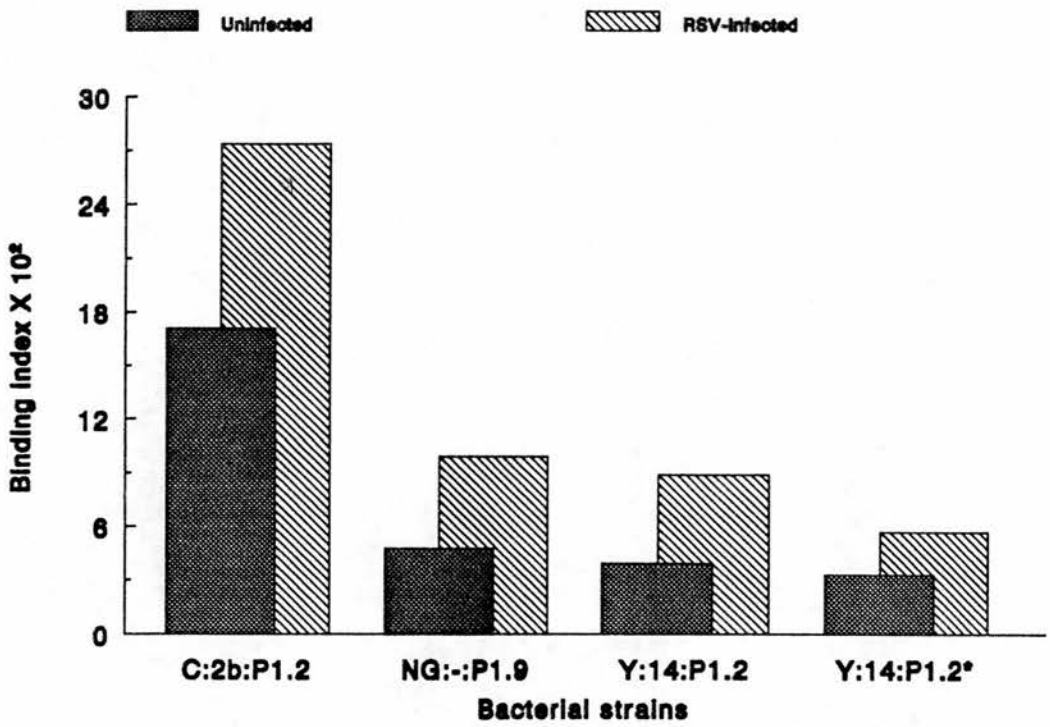

Figure 5.13. Comparison of cumulative binding of meningococcal strains to HEp-2 cells and HEp-2 cells infected with RSV at M.O.I. 1.0 at doubling ratios ranging from 40 to 640 bacteria:cell.(* pilate strain)

5.3.6. FITC labelling of HEp-2 cells

The possibility that FITC molecules dissociated from FITC-labelled bacteria and bound to RSV-infected cells more effectively so that they were more fluorescent compared with uninfected HEp-2 cells was tested.

HEp-2 cells and the cells infected with RSV at M.O.I. 1.0 were incubated with 2 dilutions (1/4 and 1/8) of the FITC solution in DPBS for 30 min at 37^0C. Fluorescent cells were detected by flow cytometry. FITC bound to a significant proportion of cells but at a very low mean fluorescence compared with the mean obtained with FITC-labelled bacteria. There was also no significant difference in the labelling detected for uninfected and RSV-infected cells (Fig 5.14).

5.4. DISCUSSION

5.4.1. Method for assessment of bacterial binding

The bacteria responsible for meningitis, (*N. meningitidis* and *H. influenzae*, bind to non-ciliated pharyngeal epithelial cells [Stephens *et al.*, 1983; Read *et al.*, 1991]. Use of the HEp-2 cell line for study of bacterial binding is relevant as HEp-2 cells are non-ciliated epithelial cells of human origin. This cell line provides a consistent source of cells for binding studies.

A variety of strains for each bacterial species with different phenotypes was used in the assays. Among the viruses affecting the respiratory tract, RSV was chosen because of the epidemiological coincidence between the diseases caused by the two types of pathogen .

Microorganisms grown in enriched media might differ in many properties from strains growing in natural ecological system [Isaacson, 1980]. Bacteria growing in a

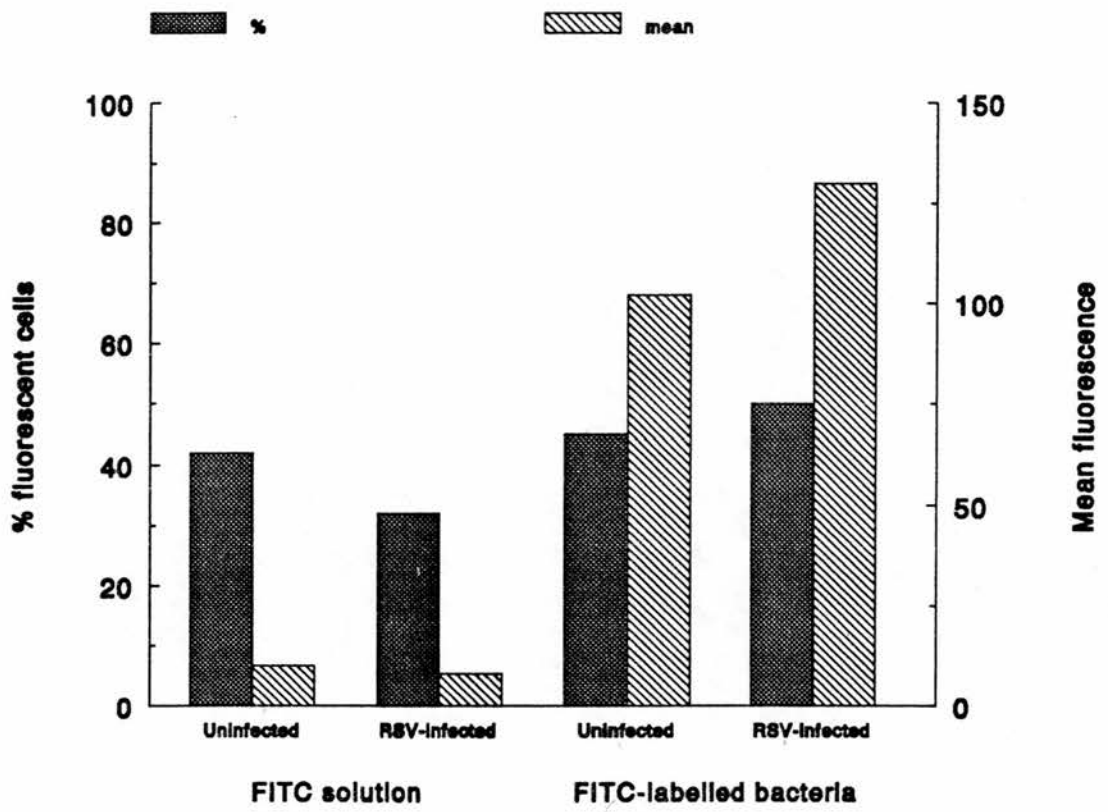

Figure 5.14. Comparison of fluorescence of uninfected and RSV-infected HEp-2 cells incubated with FITC-solution with that of cells incubated with FITC-labelled bacteria at a ratio of 80:cell.

closed system are exposed to a continually changing environment in contrast to the *in vivo* environment. Use of systems of bacterial growth in media that provide conditions which are more like those encountered in the host might be more relevant to *in vivo*. In the present binding studies, however, consistent results were obtained with bacteria grown on different media suggesting that the binding was apparently not affected by the media used.

Repeated cultures also affect bacterial pili. Pilate strains in general have been shown to bind more effectively to epithelial tissues compared with non-pilate strain. Pili can easily penetrate mucus and the epithelial glycocalyx. Pili have been considered to be responsible for 'initial' attachment to the target tissues and maintenance of a steady state of bacterial colonization. Once this has occurred in susceptible hosts, non-pilate progeny might adhere through different adherence mechanisms to obtain a closer contact with the target tissue leading to invasion. The bacterial strains used in the present study were non-pilate except for a pilate form of Y:14:P1.2.

Fluorescein isothiocyanate (FITC) was used to label bacteria in this study. FITC molecules bind to surface proteins non-specifically. The dye did not interfere with the binding as FITC-labelled bacteria bound to both HEp-2 cells and RSV-infected HEp-2 cells. The adhesion was not mediated through FITC as the dye consists of monomeric molecules. Adhesion to cells by FITC molecules that dissociate from labelled bacteria during incubation with the cells was not involved in enhanced fluorescence of RSV-infected cells (Fig 5.14). Similar results showing increased binding of bacteria to RSV-infected cells were obtained with another fluorescent dye (rhodamine) used to label bacteria in one experiment (5.3.3.1). The pattern of increased bacterial binding to RSV-infected cells compared with un-infected cells was also observed with HEp-2 cell monolayers incubated with un-labelled bacteria. These observations indicate that FITC was not involved in bacterial binding.

Airborne droplets from patients with bacterial disease of the size of >10 um that might be deposited on nasopharyngeal mucosal cells [Mims, 1987] can carry from 20 to many hundreds of bacteria. Ratios used in the assays ranging from 40 to 1000 bacteria/cell are comparable with *in vivo* conditions. Similar ranges have been arbitrarily selected for use in bacterial binding studies by other workers [St. Geme and Falkow, 1990; Blackwell *et al.* in press].

Meningococci attached more efficiently to RSV-infected cells compared with uninfected cells as assessed with the monolayer technique. The method was, however, complicated by the tendency of bacteria to form clumps making counting difficult. Bacterial counting was further complicated as both cells and bacteria stained red. The technique was discarded due to variations in the two counts made on the same samples and limited number of experiments that could be examined. It was, however, useful for determination of the optimal incubation period before cytotoxic effects occurred. These effects might be due to release of lipooligosaccharide from the bacteria [Stephens *et al.*, 1987] or the direct effect of the bound bacteria [Stephens, 1989].

Measuring fluorescent-labelled bacteria on the surface of cells by flow cytometry is a more sensitive and precise method than microscopy. More cells can be counted in a short period of time and there is no subjectivity in the values recorded.

5.4.2. Variations in the technique

There were significant day-to-day variations in the results of binding assays which can be ascribed to a number of factors.

(1) RSV is characterized by biological and biochemical instability and pleiomorphism. Amplified production of defective interfering (DI) particles of RSV by its repeated passages interferes with the standard virus in its infectivity

(von-Magnus effect). These factors can result in variable infection of cells in the samples infected at the same M.O.I.

(2) Variable degree of relative resistance of HEp-2 cells to RSV infection was noted by fluctuations in the titre of a standard virus preparation [Kim *et al.*, 1973]. Using immunofluorescence, variations in the expression of F and G glycoproteins on the surface of infected cells from samples infected at the same M.O.I. were found in the present study (Fig 5.15). Day to day fluctuations in the binding of bacteria with aliquots from a stock sample of buccal-epithelial cells have been observed [Tramont, 1977].

(3) Cells at the same growth phase might express different surface antigens. In any single preparation, the number of bacteria adhering to HEp-2 cells showed great variations; some cells bound many more bacteria than others. Phasic variations in the cells have also been demonstrated. Hynes and Bye [1974] noted a very much enhanced expression of a large glycoprotein on the surface of hamster fibroblasts arrested in the early growth phase either because of high cell population density or serum starvation. Although the cells used in the present study were grown and harvested under uniform conditions, batch to batch variations in the cells might be another factor reponsible for difference in results of binding experiments.

(4) Measurement of the bacterial concentrations by optical density (OD) can be variable. The dispersal of clumps in bacterial suspensions can vary which might affect the OD reading. To examine the variation, counts were determined by light microscopy and by the optical density method on duplicate suspensions of 5 strains of *H. influenzae* used in the assays. The correspondence in readings between the two methods was not consistent (Tab 5.3). Variations in readings by the OD method were also indicated when, in some experiments, the counts were checked by flow-cytometry at a later time (results not shown).

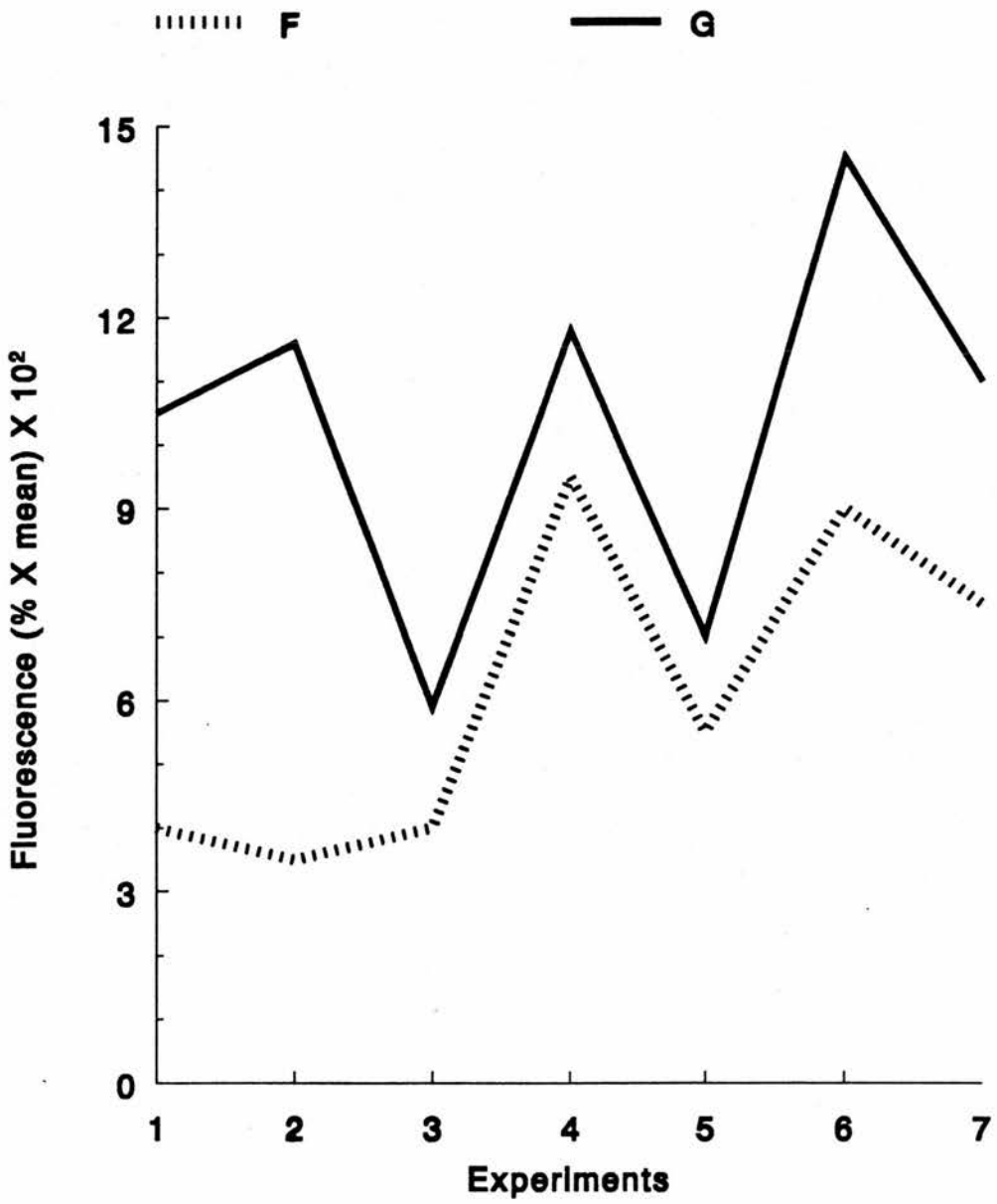

Figure 5.15. Variation in expression of F and G glycoproteins on the surface of RSV-infected HEp-2 cells assessed by flow cytometry.

Table 5.3. Difference between two counts of suspensions of 5 strains of *H. influenzae* at optical density (OD) 0.140 determined by light microscopy.

Count X 10^8/ml

Strain	1st count	2nd count
H14	3.0	3.6
H20	1.4	2.7
H21	3.6	8.4
H25	5.2	10.2
H29	2.8	5.9

(5) Application of a shear force can reduce the number of bacteria attached to cells. A 30 fold increase in the force resulted in 70-80% reduction of attached bacteria [Christersson *et al.*, 1988]. Variation in force used to disperse cells at each washing could result in detachment of variable proportions of bacteria [Rasanen, 1981].

(6) Detection of particles by the flow cytometer involves fine adjustments in the alignment of the laser beam and sample stream with the light sensors. Slight changes in the settings can affect the results. Changes in the cell suspension or cell flow rate in the flow cytometer can also affect the results. In all these experiments, the cell suspensions were kept at a constant starting concentration of $1X10^6$/ml. The cells in suspensions were thoroughly dispersed to avoid clumps. The sample flow in flow cytometer was also kept at approximately the same rate.

Most of these factors can affect the inter-experimental comparisons of results, a point to be considered in statistical analysis. Factors such as mechanical shearing which can cause intra-experimental variations, can be standardized by using uniform techniques.

5.4.3. Binding to uninfected cells and RSV-infected cells assessed by flow cytometry

In cell samples with <10% infected cells (M.O.I. 0.001), meningococcal binding was significantly decreased compared with uninfected cells; this was not anticipated. Internalization of *Salmonella typhimurium* by HEp-2 cells was inhibited by pretreatment of cells with tissue necrosis factor (TNF) or TNF and interferon (IFN) α or γ [Degre *et al.*, 1989]. Similarly, treatment of primary chicken kidney cultures with the INF-γ caused inhibition of attachment by sporozoites of *Eimeria tenella* to the cells [Kogut and Lange, 1989]. The RSV infected cells might be secreting soluble factors such as IFN which inhibit binding of meningococci to neighbouring uninfected cells in the sample. Compared with controls, total bacterial binding was

not significantly altered for the samples containing 20-30% infected cells (M.O.I. 0.01). This might reflect the mixed population of infected and uninfected cells. The effect of enhanced binding to the infected cells might be masked by decreased binding to the neighbouring uninfected cells mediated by the soluble factors.

Enhanced binding of meningococci, Hib and staphylococci to cells infected with RSV at M.O.I. $\geq$ 0.1 24 hr post-infection suggests four possibilities: 1)RSV infection might be enhancing the expression of existing receptor(s) [Hynes and Bye, 1974]; 2) The F or G glycoproteins of RSV expressed on the infected cells might act as additional receptors for the bacteria [Sanford *et al.*, 1978]; 3) It might be inducing expression of a new cell receptor for the bacteria [Adachi *et al.*, 1988; Okada and Tsuji, 1990]; 4) RSV infection is associated with appearance of branching filaments on the infected cells [Parry *et al.*, 1979] which might physically trap the bacteria. Hyoptheses 1 and 2 are tested in chapter 6.

Changes in the surface antigens of HEp-2 cells due to RSV-infection

6.1. Introduction

6.1.1. Blood group antigens and bacterial binding

The evidence that HEp-2 cells infected with RSV bind bacteria more effectively compared with uninfected cells (Chapter 5) suggests that viruses might (1) enhance existing bacterial receptor(s) (2) provide additional receptors in the form of viral glycoproteins (3) induce expression of new receptors and/or (4) help trap bacteria by physical changes produced on the infected cells.

The higher amounts of Le^a antigen on cells of non-secretors has been suggested to contribute to susceptibility to disease due to *H. influenzae*, meningococci [Blackwell *et al.*, 1986a, b], staphylococci [Saadi, submitted for publication] and *Candida* species [May *et al.*, 1989; Aly, 1992]. Expression of Lewis and H blood group antigens on HEp-2 cells was assessed in chapter 4.

The Lewis antigens, the *in vivo* source of which is largely obscure, are normally adsorbed on the epithelial surface from the secretions. Viral infections have been reported to cause changes in the cellular metabolism and surface expression of a variety of antigens [Hynes and Bye, 1974; Peluso *et al.*, 1978; Caldwell *et al.*, 1988; Adachi *et al.*, 1988]. HIV has been shown to induce expression of blood groups antigens on cells normally devoid of these antigens [Arendrup *et al.*, 1991]. RSV infection stimulates glycosylation of macromolecules in HeLa cells [Levine *et al.*,

1977] indicating increased enzyme activity. In the first phase of this study, the hypothesis that higher amounts of Le^a, in contrast to Le^b or H antigens, might be expressed on RSV-infected cells compared with uninfected cells was tested.

6.1.2. Viral glycoproteins and bacterial binding

In the second phase, the hypothesis tested was that the F and/or G glycoproteins of RSV expressed on the surface of infected cells might act as additional receptors for the bacteria.

6.1.2.1. F and G glycoproteins of RSV

RSV contains a single genome encoding 10 proteins, L (250K), G (84K), F (70K), N (43K), P (27K), M (28K), M_2 (22K), SH (13-30K), IC (15K) and IB (14K). The seven larger proteins have been reported to be structural and four of them, G,F,M and 24K, are related to the virus envelope [Huang *et al.*, 1985]. Little has been reported about the structure and function of the M and 24K surface proteins while G and F glycoproteins are regarded as attachment [Levine *et al.*, 1987] and fusion proteins respectively [Walsh and Hruska, 1983].

The majority of the mature form of the G glycoprotein (85-90K) consists of monomeric molecules with a 36K polypeptide chain of 298 amino acids [Wertz *et al.*, 1985; Satake *et al.*, 1985]. A dimeric form of 175K is also found [Lambert, 1988]. The G glycoprotein is secreted by infected cells in a soluble form which is shorter than the native protein by about 60 amino acids [Hendericks *et al.*, 1987].

The 70K F glycoprotein is composed of two fragments of approximately 50K (F1) and 20K (F2) having a polypeptide of 574 amino acids [Collins *et al.*, 1984].

G glycoprotein of RSV is unusual among viral glycoproteins in that it is heavily

O-glycosylated and has additional potential sites for O-glycosylation [Lambert, 1988]. The glycoprotein does not have neuraminidase or haemagglutination activities characteristic of the attachment proteins of other members of the Paramyxoviridae family [Richman *et al.*, 1971]. F glycoprotein of all the members of Paramyxoviridae is similar in structure and function.

The two glycoproteins are expressed on the surface of RSV-infected cells and can be detected with specific antibodies.

6.1.3. Objectives

The objectives of this part of the study were:

(1) to compare the effects of pre-treatment of RSV-infected cells with monoclonal anti-F or anti-G on bacterial binding;

(2) to assess the effect of pre-incubation of RSV-infected cells with bacteria on binding of anti-F or anti-G;

(3) to demonstrate viral attachment to bacteria and to compare the effect of anti-F and anti-G on viral attachment of the virus to bacteria.

6.2. Materials and Methods

6.2.1. Effect of RSV-infection of HEp-2 cells on detection of blood group antigens

Fluorescence microscopy and flow cytometry were used to detect the blood group antigens on HEp-2 cells and HEp-2 cells infected with RSV at M.O.I. 1.0 24 hr post infection. The methods are described in detail in chapter 4. Some monolayers and samples of cells in suspensions were assessed in parallel for the presence of RSV.

6.2.2. Viral antigens and bacterial binding

Different experimental approaches were used to examine the role of viral glycoprotein in bacterial binding. All the incubations in the experiments were performed at 37^0C with gentle rotation at 60 rpm with an orbital incubator (Gallenkamp) unless otherwise indicated. FITC-labelled *N. meningitidis* (C:2b:P1.2) (2.6.4) grown on BBA and HEp-2 cells and HEp-2 cells infected with RSV at M.O.I. 1.0 24 hr post-infection were used in these assays.

6.2.2.1. Absorption of antibodies

In some experiments monoclonal antibodies absorbed with HEp-2 cells were used. The antibodies (200 ul, 1/2 in PBS) were incubated with a pellet of $6X10^6$ cells overnight at 4^0C with gentle end to end rotation. The supernatant was stored in aliquots at -20^0C.

6.2.2.2. Effect of monoclonal anti-F and anti-G antibodies on binding of bacteria to HEp-2 cells

The method described in section 5.2.4 was used with a few modifications. HEp-2 cells or RSV-infected HEp-2 cells (1 X 10^7/ml MM) (1 ml) were incubated with un-absorbed or absorbed preparations of monoclonal anti-F or anti-G (1/200) for 30 min. After washing 3 times with DPBS to remove unbound antibodies, the cells were adjusted at 1 X 10^6/ml and incubated in 200 ul volumes with equal volumes of FITC-labelled bacteria at a ratio of 160 bacteria:cell for 30 min. The cells were washed 3 times, suspended in 200 ul of PBS and fixed with 100 ul of 1% buffered paraformaldehyde. The samples were analysed by flow cytometry for the percentage of fluorescent cells and the mean fluorescence.

6.2.2.3. Effect of bacterial binding on the binding of anti-F or anti-G antibodies to RSV-infected HEp-2 cells

The cells (200 ul at a concentration of $1X10^6$/ml) were mixed with un-labelled bacteria at a ratio of $2X10^3$ bacteria:cell for 15 min. Longer incubations at this ratio caused toxicity to cells. After 3 washings with DPBS, the cells with or without bound bacteria were incubated with monoclonal anti-F or anti-G (un-absorbed) at a dilution of 1/100 for 30 min. The cells were washed 3 times, suspended in 100 ul PBS and incubated with FITC-labelled anti-mouse immunoglobulin antibodies (1/200 in PBS) for 30 min. After 3 washings and fixing, the samples were assessed by flow cytometry.

6.2.2.4. Determination of viral attachment to bacteria and effect of antibodies on the attachment

The viral suspensions used in these assays contained $1X10^6$ infectious particles/ml. The bacterial suspensions were adjusted to $1X10^5$/ml. The monoclonal anti-F and anti-G used in the initial assays were unadsorbed and preparations adsorbed with HEp-2 cells were used in the latter assays. The combinations of viruses, bacteria and antibodies examined are illustrated in Table 6.1.

Different incubations carried out are described in the following sections. After incubations, all the bacterial samples were washed 3 times and mixed with FITC-labelled anti-mouse immunoglobulin antibodies (Sigma) (2.5.3) (1/200) for 30 min. The samples were washed 3 times and the pellets were resuspended in 100 ul of DPBS and fixed with 50 ul of 1% paraformaldehyde. The bacteria were analysed by flow cytometry for the percentage of fluorescent bacteria and the mean fluorescence.

6.2.2.4.1. Incubation of viruses and bacteria with antibodies

Aliquots of viral suspensions or HEp-2 cell lysate (300 ul) were incubated with

Table 6.1. Incubations of bacteria with RSV (RSV-infected cell lysate), HEp-2 cell lysate, monoclonal anti-F or anti-G and anti-mouse immunoglobulin antibodies during assays of virus attachment to bacteria and inhibition of attachment.

Bacteria	+	-	+	+	+	-
Bacteria + anti-F or anti-G	-	+	-	-	-	+
Virus or cell lysate	-	-	+	+	-	+
Virus or cell lysate + anti-F anti-G	-	-	-	-	+	-
Anti-F or anti-G	-	-	-	+	-	+
FITC-labelled anti-mouse immunoglobulin	-	+	+	+	+	+

+ = added

- = not added

different dilutions of monoclonal anti-F or anti-G for 60 min. Bacterial suspensions (300 ul) (1 X 10^8/ml) were similarly incubated with the antibodies for 30 min and washed 3 times with DPBS by centrifugation at 2500 g for 10 min. The bacterial suspensions were adjusted at 1 X 10^5/ml. The antibody-treated preparations of viruses or bacteria were used in the latter part of the experiment.

6.2.2.4.2. Virus attachment to bacteria

Samples (30 ul) of bacterial suspensions or bacterial suspensions treated with either of the antibodies (6.2.2.4.1) were mixed with 300 ul of cell lysate or virus suspension to provide a ratio of 100 infectious particles (p.f.u.)/bacterium for 30 min. After washing 3 times, the bacterial preparations were incubated with anti-F or anti-G at a dilution of 1/100 for 30 min.

6.2.2.4.3. Inhibition of viral attachment

To examine the blocking effect of the two antibodies, the bacteria (1 X 10^5/ml MM) (30 ul) were incubated for 30 min with 300 ul of cell lysate or virus suspension (100 p.f.u./bacterium) that had been treated with either of the two antibodies at different dilutions (6.2.2.4.1).

6.2.2.5. Statistical methods

No formal statistical analysis could be performed on the data from the assays examining the role of antibodies for experiments outlined in section 6.2.2.2 and 6.2.2.4 because too few replicate experiments were done. The number of experiments were limited by the small supply of monoclonal anti-F and anti-G antibodies. A paired 't' test was done on logs of binding indices obtained from each sample in

experiments examining the effect of bacterial binding on binding of anti-F or anti-G.

6.3. Results

6.3.1. Effect of RSV-infection of HEp-2 cells on detection of blood group antigens

6.3.1.1. Fluorescence microscopy

The monolayers infected with RSV (M.O.I. 1.0) 24 hr post-infection showed about 80% fluorescent cells when tested with monoclonal antibody to F glycoprotein of RSV. RSV infection did not change the patterns of binding of monoclonal antibodies to Le^a, Le^b, H-type 2 or FITC-UEAI on HEp-2 cells (4.3.1.1).

6.3.1.2. Flow-cytometry

The data from 4 experiments assessed by flow cytometry using indirect immunofluorescent techniques suggested that RSV infection of the cells did not change the binding of antibodies or UEAI to HEp-2 cells (Fig 6.1). Fluorescence on cells with anti-Le^a could only be expressed as percentage of fluorescent cells as means for small percentages cannot be obtained.

6.3.2. Viral antigens and bacterial binding

6.3.2.1. Effect of monoclonal anti-F and anti-G antibodies on binding of bacteria to HEp-2 cells

Results from 5 replicate experiments indicate that pre-treatment of both uninfected and RSV-infected cells with monoclonal anti-F or anti-G (both at 1/100) reduced the bacterial binding. The pattern of increased bacterial binding to RSV-infected cells

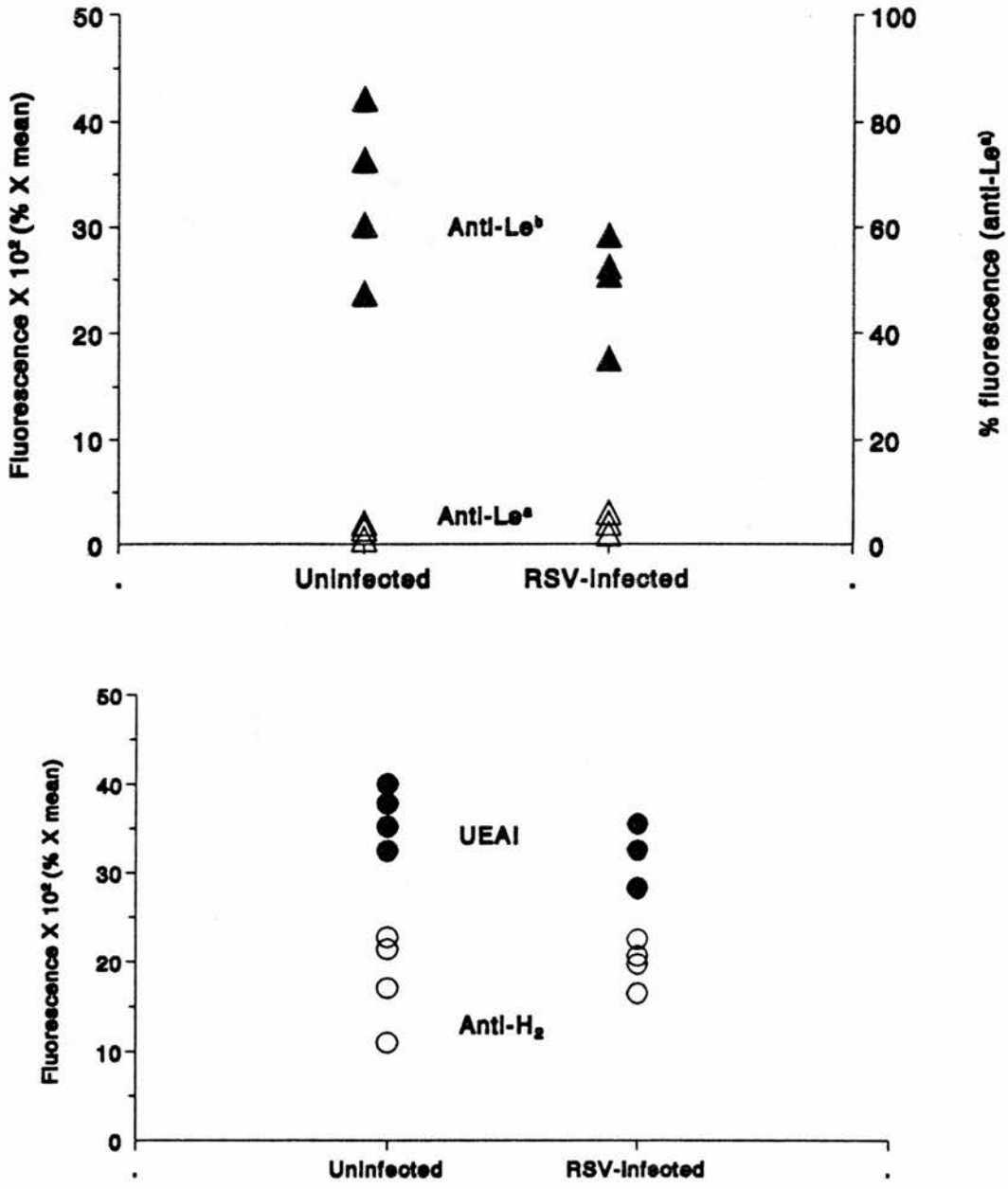

Figure 6.1. Effect of RSV-infection of HEp-2 cells on binding of monoclonal antibodies to Lewis and H-type 2 antigens and FITC-labelled UEAI. An indirect immunofluorescence technique was used for antibody assay.

compared with uninfected cells was not altered (Fig 6.2). The antibody preparations were adsorbed with HEp-2 cells in an attempt to reduce non-specific effects. Results from 4 repeated experiments with adsorbed preparations (1/100) indicate that anti-F did not affect binding to uninfected HEp-2 cells, but anti-G increased the binding to these cells. The reasons for this increase could not be explained; but antibody molecules were not likely to be involved since the antibodies could not be detected on uninfected cells by flow cytometry. Both the antibodies decreased bacterial binding to RSV-infected cells but the effect appeared to be more pronounced with anti-G (Fig 6.2).

6.3.2.2. Effect of bacterial binding on binding of anti-F and anti-G to RSV-infected HEp-2 cells

Results from 7 experiments indicate that both the antibodies bound to the viral glycoproteins on the surface of infected cells, but the mean fluorescence recorded on the cells incubated with anti-G was higher compared with anti-F ($P<0.05$).

Significantly lower levels of mean fluorescence were recorded for anti-G with RSV-infected cells pre-treated with bacteria compared with the infected cells that had not been treated with bacteria ($P<0.01$). This effect was not observed with anti-F (Fig 6.3). Amounts of fluorescence detected on cells in the experiments with anti-G corresponded to a dose-response effect with ratios of bacteria:cell ranging from 500 to 4000 (Fig 6.4).

6.3.2.3. Viral attachment to bacteria and the role of antibodies in inhibition of the attachment

RSV attached to meningococci in 12 repeated assays. Both the antibodies, in unabsorbed state, incubated with viruses at a dilution of 1/100 increased attachment

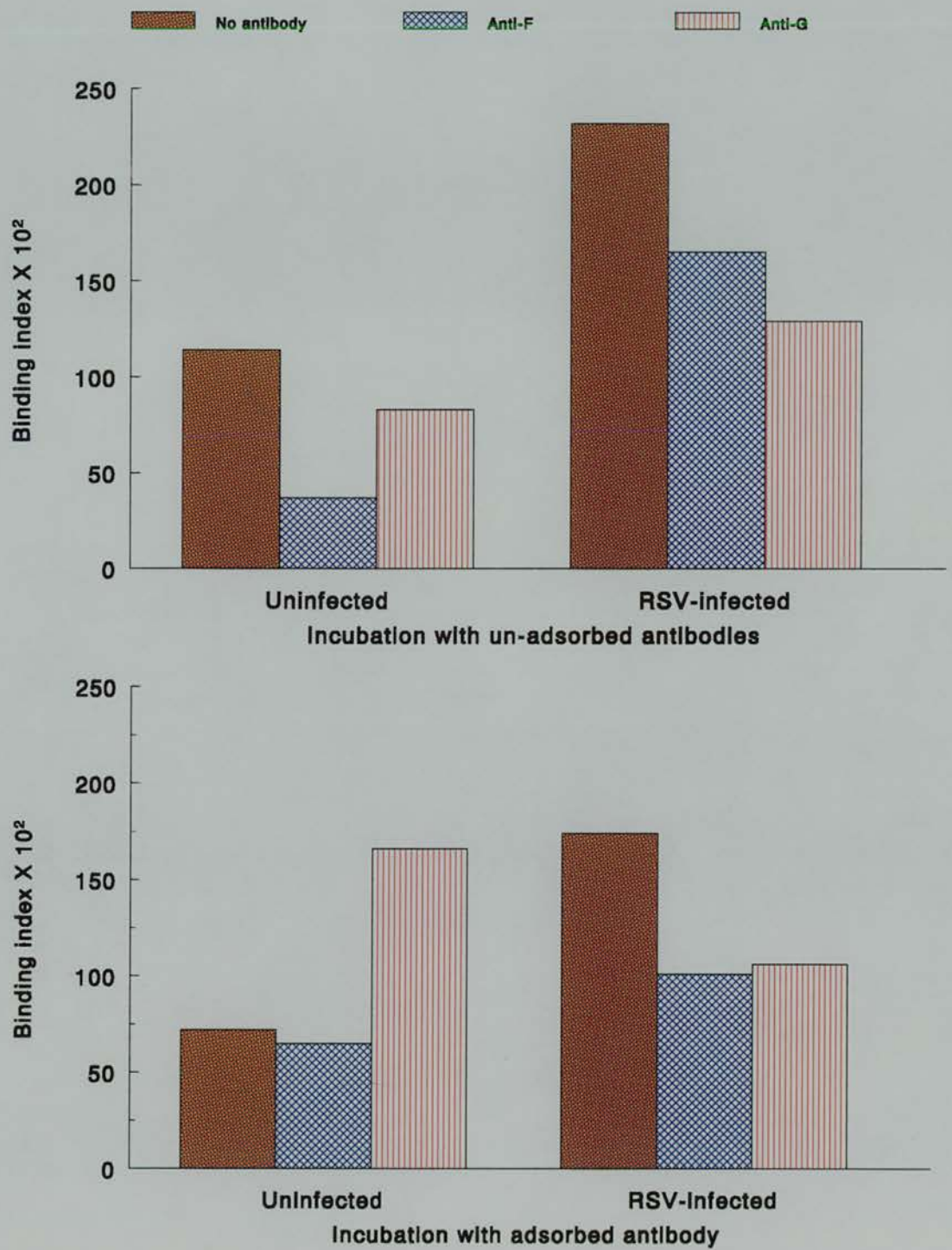

Figure 6.2. Effect of incubation of HEp-2 cells and RSV-infected HEp-2 cell with different preparations of monoclonal anti-F or anti-G antibodies on binding of fluorescein-labelled meningococci.

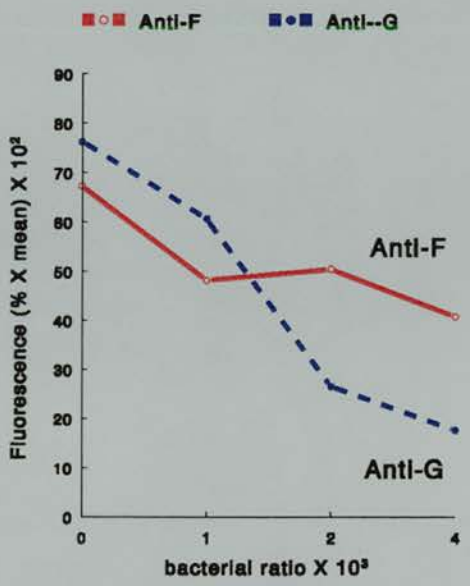

Figure 6.4. Dose-response effect of incubation of RSV infected HEp-2 cells with bacteria on detection of F and G glycoproteins.

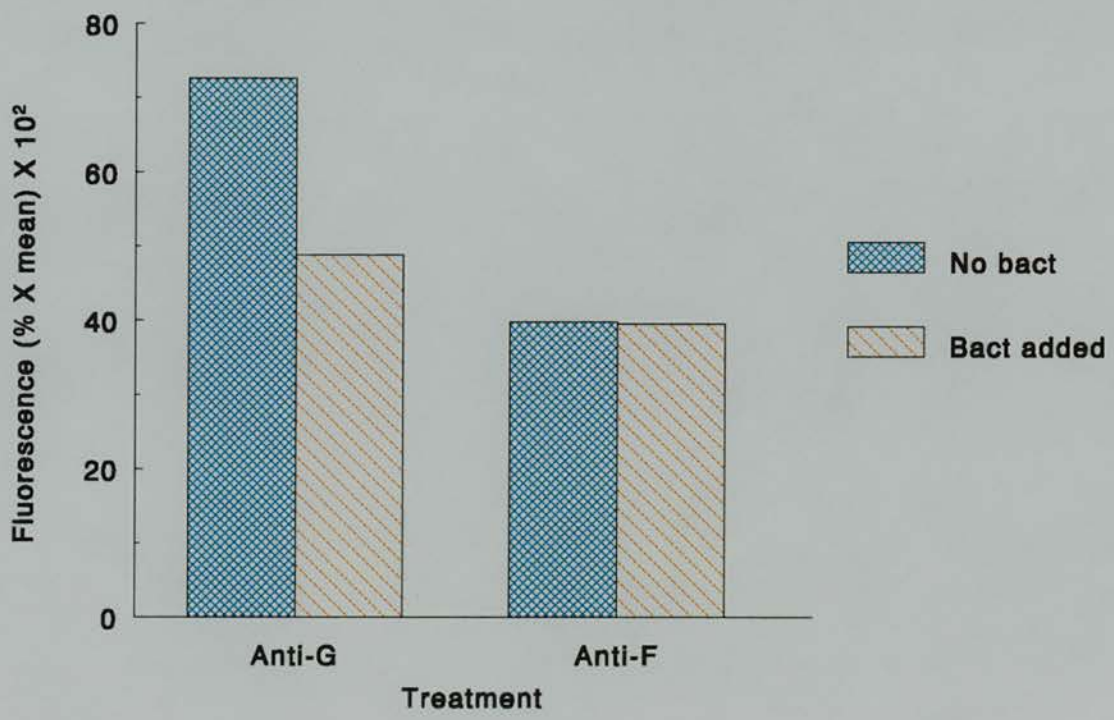

Figure 6.3. Average effect of incubation of RSV-infected HEp-2 cells with meningococci at a ratio of $2X10^3$ bacteria:cell on detection of F and G glycoproteins observed in 7 replicate experiments.

of the viruses to bacteria in 4 experiments. Results from 4 assays using adsorbed preparations at dilution 1/200 showed that anti-F did not alter the viral attachment to the bacterium while anti-G reduced the attachment greatly (Fig 6.5). The adsorbed antibodies used at dilution 1/100 gave variable results (not shown).

The antibodies incubated with bacteria had variable effects on subsequent viral attachment; monoclonal anti-F reduced the viral attachment while anti-G increased it. The non-specific effect of antibody preparations on viral attachment was partially reduced by absorption of the antibody preparations with HEp-2 cells and by the use of more diluted preparations of antibodies (1/200). When incubated with bacteria, the adsorbed preparation of anti-F still reduced the subsequent viral attachment while anti-G did not greatly affect the attachment (Fig 6.6).

6.4. Discussion

RSV infection did not change the binding of UEAI or monoclonal antibodies to Le^a, Le^b or H-type 2 antigens on the cells. The hypothesis that enhanced expression of Le^a antigen might be associated with increased binding of the bacteria on RSV-infected HEp-2 cells was disproved.

Antibodies to viral glycoproteins have been used in studies of inhibition of bacterial binding to receptors on virus-infected cells [Sanford *et al.*, 1978]. Use of monoclonal antibodies in these assays is limited as they might not be directed to an epitope of the molecule interacting with bacterial adhesins. Preparations of polyclonal antibodies or a combination of monoclonal antibodies directed to different epitopes on the proposed receptor molecule might be more useful in studies to identify a receptor.

The results of the assays in which bacterial binding was assessed after the cells were treated with anti-F or anti-G were difficult to interpret because the treatment enhanced the bacterial binding to uninfected HEp-2 cells. These effects could be due

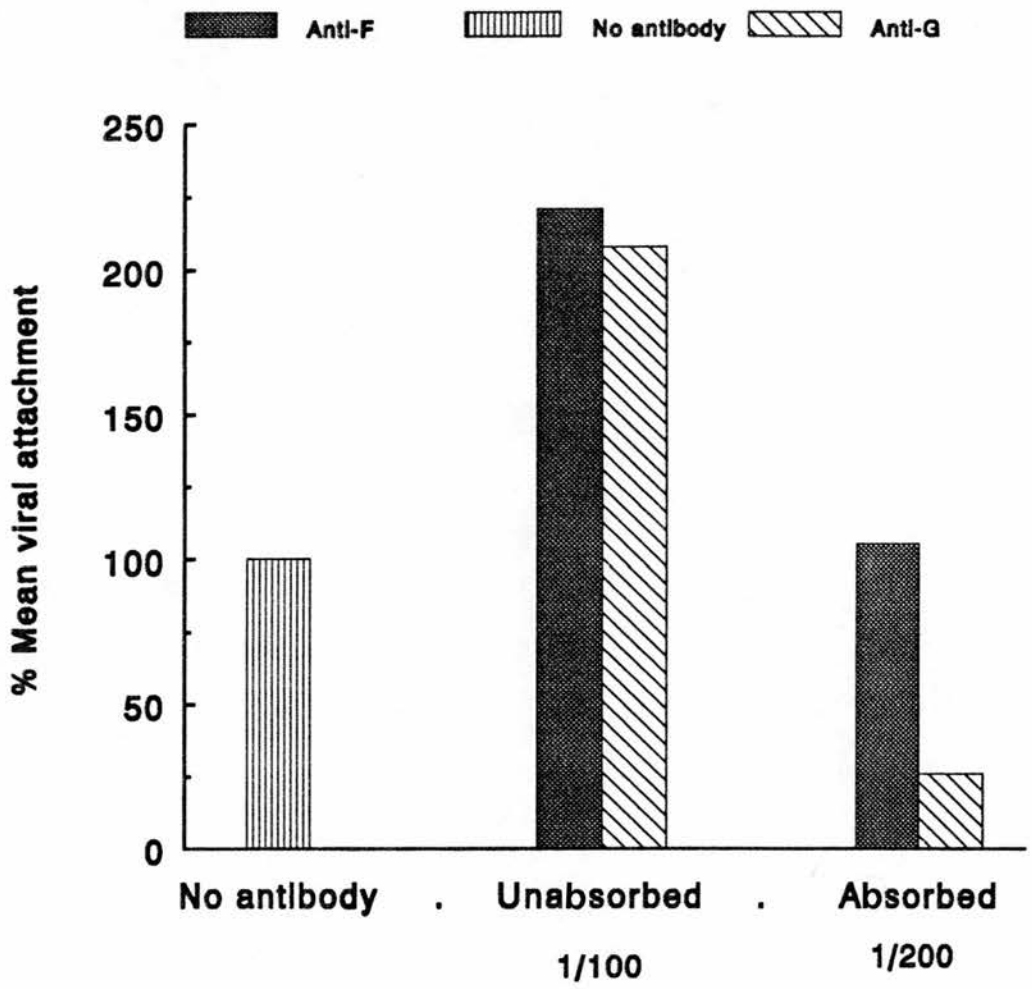

Figure 6.5. Effect of incubation of RSV with different preparations of monoclonal anti-F or anti-G antibodies on virus attachment to meningococci (12 experiments).

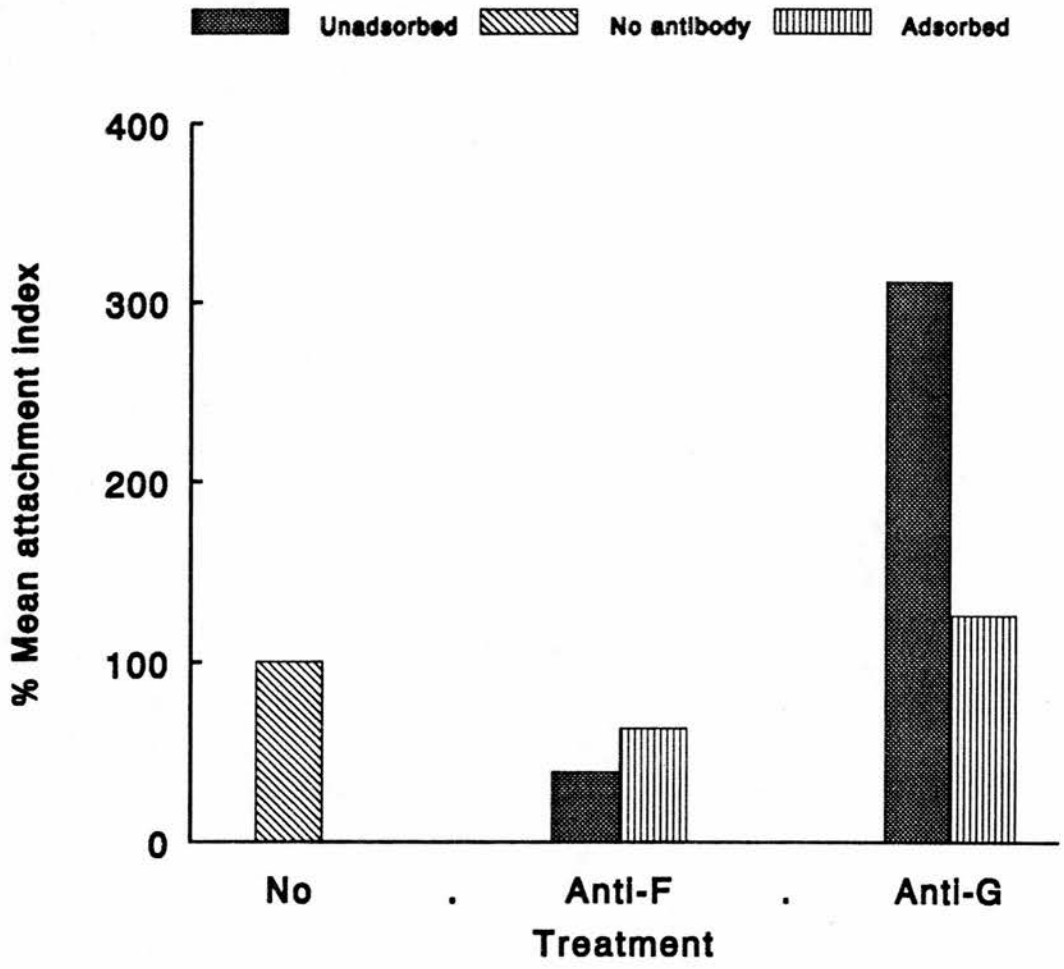

Figure 6.6. Effect of incubation of bacteria with unadsorbed or adsorbed anti-F or anti-G antibodies on attachment of RSV to bacteria (8 experiments).

to substances other than antibodies present in the ascitic fluid. The non-specific effects were partially removed by adsorption of the preparations with HEp-2 cells. The antibodies could not be purified by immunoaffinity because the purified F and G glycoproteins were not available.

There was some evidence that anti-G inhibited bacterial binding to RSV-infected cells (Fig 6.2). On the other hand, the presence of bacteria on the cell surface significantly reduced the binding of anti-G (Fig 6.3). It is not clear if the decrease in binding of anti-G was due to binding of bacteria to the epitope specific for the antibody idiotype or due to steric hindrance.

The evidence of viral attachment to the bacteria also suggests that viral surface proteins might be involved in bacterial binding. The attachment of viruses to bacteria could be reduced by the adsorbed preparation of anti-G but not by anti-F antibodies. The increased attachment of viruses with higher concentrations of antibodies in some experiments might be due to attachment of large aggregates of viruses in antigen-antibody complexes. This could also be due to non-specific effect on bacteria due to substances present in the antibody preparations such as products of inflammatory activity in the ascitic fluid used as the source of antibodies. The non-specific effect could not be completely removed by adsorption with HEp-2 cells. Adsorption with bacteria would be preferable but it was not possible because of limited amount of the monoclonal anti-F and anti-G antibodies.

In conclusion, the results presented here indicate that the surface glycoprotein G contributes to the increased adhesion of meningococci to RSV-infected HEp-2 cells.

Effect of modification of cell surface carbohydrates on bacterial binding to HEp-2 cells

7.1. Introduction

Many receptors for bacteria on host epithelial surfaces are carbohydrates. Adhesins of *Vibrio cholerae* appeared to bind to D-mannosyl and L-fucose receptors [Jones and Freter, 1976]. Work of Ofek and Beachey [1978] and Leffler and Swanborg-Eden [1980] revealed sugars, galactoside and D-mannosyl, involved in binding of *E. coli.* Sialic acid moieties on sheep erythrocytes were identified as receptors for *Mycoplasma pneumoniae* [Feldner *et al.*, 1979]. Attachment of a variety of Gram-positive and Gram-negative organisms to mouse peritoneal-exudate macrophages was inhibited by glucose and galactose [Freimer *et al.*, 1978]. Monosaccharides, N-acetyl-D-galactosamine, D-galactose, α-methyl-D-mannoside and L-fucose, inhibited the binding of *E. coli, Bacillus subtilis* and *Bacillus melitensis* to lymphocytes. Conversely, the binding of *Staph. aureus* to these cells was increased in the presence of N-acetyl-D-galactosamine [Rasanen, 1981]. *H. influenzae, N. meningitidis* and *Staph aureus* were found not to possess mannose-binding activity [Mirelman *et al.* 1980]. The effects of other monosaccharides were not included in their study.

7.1.1. Chemical nature of G and F glycoproteins of RSV

G glycoprotein of RSV consists of 50-60% sugars. The polypeptide contains 298 amino acids over 30% of which are hydroxy-amino acids, serine and threonine

[Wertz *et al.*, 1985; Grubber and Levine, 1985]. These amino acids provide sites for O-glycosylation. About half of the sites must be glycosylated for maturation of the molecule; the rest remain unoccupied [Lambert, 1988]. The N-terminus of the protein is intracellular. The peptide from 64-298 amino acids is predicted to be extracellular and 77 out of 91 potential sites for O-glycosylation are clustered in this region. The glycoprotein contains a high proportion of basic amino acids [Satake *et al.*, 1985] and proline residues making it a mucinous type of protein [Wertz *et al.*, 1985]. Seven asparagine residues which are potential sites for attachment of sugars through N-linkage reside near the C-terminal [Lambert, 1988].

F glycoprotein has 5 potential acceptor sites for asparagine-linked sugars (N-glycosylation) and a site for proteolytic cleavage of the molecule into F1 and F2 fragments. Carbohydrates of the molecule are completely sensitive to tunicamycin, an antibiotic which inhibits N-glycosylation. Carbohydrates, mainly located in the F-2 segment, make up 13% of its mass [Lambert, 1988].

7.1.2. Reagents used to modify carbohydrates

A variety of reagents have been employed to delete chemical groups from the surface molecules in an attempt to discover the role of these groups in bacterial binding. These include enzymes and chemicals which specifically cleave defined groupings or inhibit addition of groups to molecules.

7.1.2.1. Monensin

Monensin is a carboxylic ionophore that interferes with the membrane flow of proteins between the medial and the trans compartment of the Golgi apparatus. This blockage results in inhibition of O-glycosylation and of trimming of some other sugars bound to proteins, but N-glycosylation is not affected [Pressman, 1976].

Monensin treatment does not affect the transportation of the F and G glycoproteins to the surface of infected HEp-2 cells [Satake *et al.*, 1985].

With monensin and related enzymes, G glycoprotein has been shown to be glycosylated through both O- and N-linkages. Tunicamycin causes a slight decrease in size of the molecule. In comparison, monensin treatment results in a dramatic loss of molecular weight [Wertz *et al.*, 1985] indicating that the molecule is mainly O-glycosylated. Monensin also significantly reduces the yield of the infectious virus particles. Yield of RSV from HeLa cells was inhibited by 99.6% when the infected cells were treated with monensin (0.1 uM) 2 hr post-infection [Gruber, 1985].

7.1.2.2. Periodate

Sodium periodate ($NaIO_4$) cleaves C-C bonds and oxidizes alcohol groups to aldehydes on terminal sugars of molecules. Mild treatment with periodate has been shown to remove the terminal two carbon atoms from sialic acid while it causes minimal destruction to other sugars within molecules. Severe treatment can cause destruction of additional neutral sugar residues within oligosaccharide chains [Lenten and Ashwell, 1971]. For instance, treatment of glycopeptides obtained from human γ-globulin with 0.06 M periodate (severe treatment) resulted in total loss of sialic acid, fucose and galactose and partial loss of glucosamine moieties. This treatment also causes partial oxidation of amino acids such as tyrosine, tryptophan, cystine and methionine. In the glycopeptides from human γ-globulin, 56% of tyrosine was lost during the procedure [Rothfus and Smith, 1963].

The objective of this part of the study was to determine if the surface carbohydrate moieties of G glycoprotein were involved in the increased bacterial binding to RSV-infected HEp-2 cells.

7.2. Materials and Methods

All the incubations were carried out at 37^0C with rotation at 60 rpm in an orbital incubator (Gallenkamp) unless otherwise indicated. DPBS was used for washing during the experiments. Meningococcal strain C:2b:P1.2 grown on BBA and HEp-2 cells and HEp-2 cells infected with RSV at M.O.I. 1.0 were used for the binding studies.

7.2.1. Treatment of cells with reagents

7.2.1.1. Treatment of cells with monensin

A stock solution containing 10 mM monensin (sodium salt) (Sigma) was prepared in absolute ethyl alcohol and stored in aliquots at -70^0C for use within 3 months of preparation. For determination of toxic effects and effects on RSV infection of the cells, HEp-2 cell monolayers in 25 cm^2 tissue culture flasks were grown in medium containing monensin at 0.1 uM. Monensin-treated or untreated monolayers were infected with 200 infectious virus particles in 2 ml MM. After adsorption for 60 min, the monolayers were washed twice and kept in MM for 2 hr. The monolayers were maintained in overlay medium (without additional $NaHCO_3$) (2.5.2.1) with or without monensin for 3-4 days until cytopathic effects appeared.

The cells for bacterial binding assays were treated with monensin as follows. Monolayers of HEp-2 cells or RSV-infected HEp-2 cells 2 hr post-infection were incubated with monensin at concentrations ranging from 0.05 uM to 0.2 uM in MM [Gruber and Levine, 1985; Satake *et al.*, 1985]. The cells were harvested with EDTA 18-20 or 30 hr post-infection as described in section 5.2.4. The cell count was adjusted to $1X10^6$/ml in MM without antibiotics.

7.2.1.2. Treatment of cells with sodium periodate

Aliquots of stock solution containing sodium m-periodate ($NaIO_4$) (Sigma) at 150 uM in distilled water were stored at 4^0C. Samples of suspensions of HEp-2 cells and RSV-infected HEp-2 cells 20 hr post-infection were incubated with sodium periodate at concentration of 1 uM (mild treatment) or 7.5 uM (severe treatment) [Ofek *et al.*, 1977; Ogmundsdottir *et al.*, 1978] in MM without antibiotics for 5 or 20 min. After washing 3 times, the count was adjusted to $1X10^6$/ml in MM without antibiotics.

7.2.1.3. Treatment of cells with neuraminidase

One unit of neuraminidase (Sigma) releases up to 1.0 mM of N-acetyl neuraminic acid per minute (manufacturer's information). Aliquots of suspensions (300 ul) containing $3X10^5$ uninfected or RSV-infected HEp-2 cells were incubated with 0.1 units of neuraminidase at pH 6.0 for 20 min. The cells were washed and counted as described above.

7.2.2. Bacterial binding assays

Following the treatments described above, cells were used in binding assays with different ratios of bacteria:cell as described in section 5.2.4. Binding of fluorescein-labelled bacteria to the cells was analysed by flow cytometry as described in section 2.7.

7.2.3. Detection of viral glycoproteins on the infected cells

In parallel with the bacterial binding assays, the treated RSV-infected cells were compared with untreated RSV-infected cells for interaction with monoclonal anti-G (2.5.3.2). Monensin-treated infected cells were also assessed for effects of treatment on F glycoprotein. The effects of other treatments on F glycoprotein could not be examined due to limited supply of the monoclonal anti-F.

7.2.4. Statistical methods

The data from repeated binding experiments using cells treated with monensin (7 experiments) or periodate (7 experiments) were subjected to 'analysis of variance' carried out on logs of binding indices. The paired t-test was used to analyse the effect of the monensin or periodate on the detection of G glycoprotein. The results from binding studies in which neuraminidase was examined were not formally analysed due to small number of experiments (2 experiments).

7.3. Results

7.3.1. Effect of RSV-infection on bacterial binding

The overall effect of increased binding of bacteria at a range of ratios (80 to 1280) bacteria:cell to RSV-infected cells compared with uninfected cells was significant ($P<0.001$). This confirmed the finding from earlier binding studies (Chapter 5).

7.3.2. Results from monensin treated cells

7.3.2.1. Effect of monensin on cell morphology and RSV infection

HEp-2 cells incubated with 0.1 uM of monensin for 4 days were of unusual spindle shape with branching processes. Treatment of cells with monensin for 24 hr, however, caused only minimal histological changes but it slowed down the cell replication (Fig 7.1). Monensin greatly reduced the permissibility of HEp-2 cells to RSV infection; but, after the cells were successfully infected with the virus, treatment with monensin did not greatly affect plaque formation (Fig 7.2).

7.3.2.2. Effect of monensin on bacterial binding and detection of viral glycoproteins

Figure 7.3 summarizes the results of 7 experiments in which cells were incubated

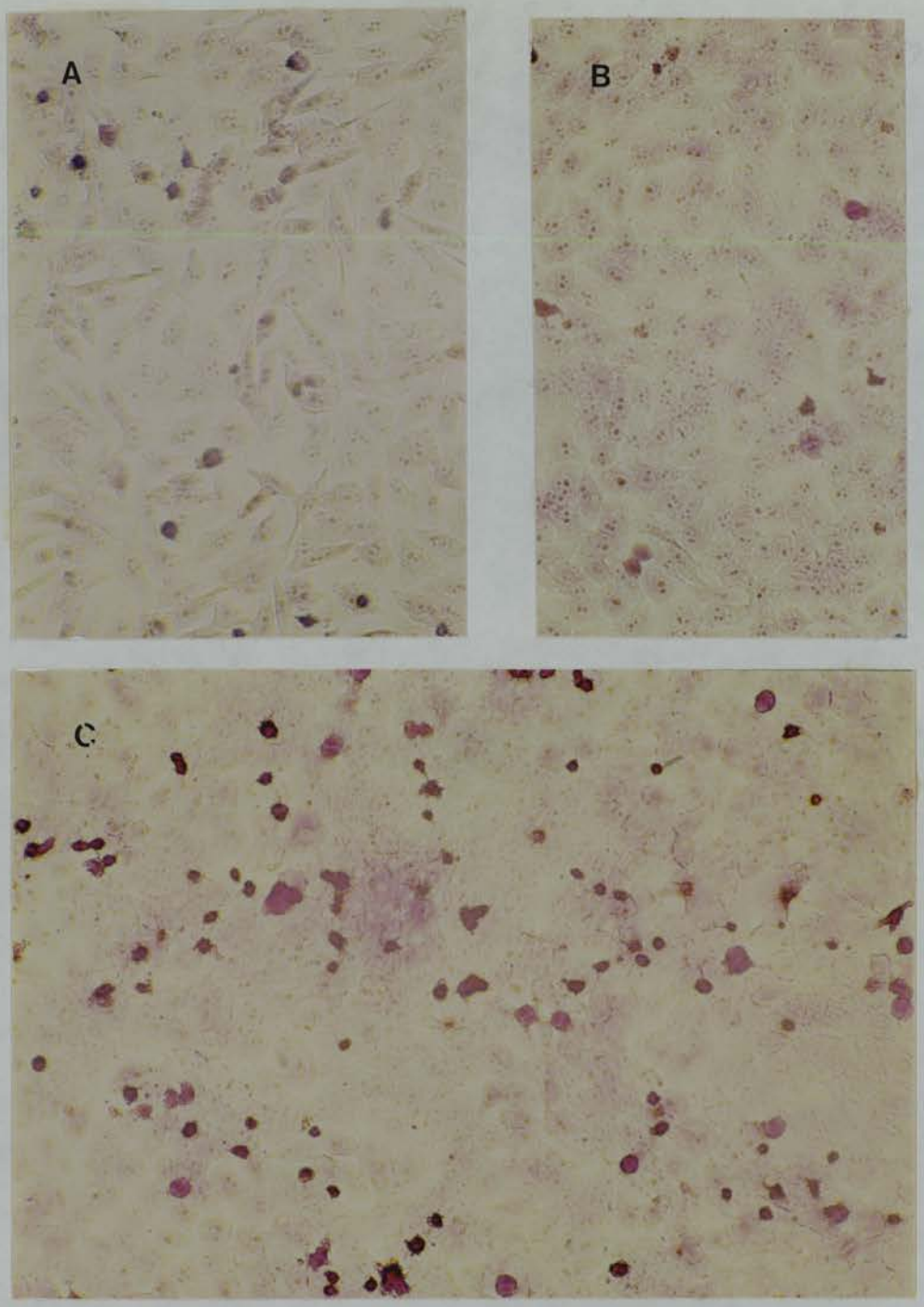

Figure 7.1. Replicate monolayers of HEp-2 cells maintained in maintenance medium (MM), MM with monensin for 24 hr(B) or MM with monensin for 72 hr (C). (magnification: X40)

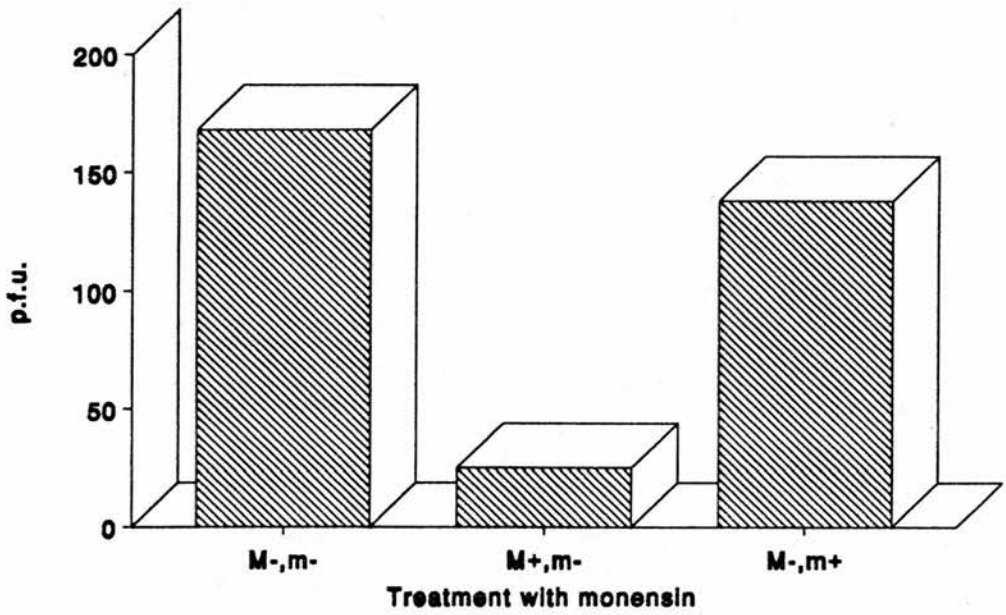

Figure 7.2. Effect of monensin on formation of plaques in HEp-2 cell monolayers by infectious particles of RSV. (M = incubation of monolayers with monensin during pre-infection period and m = during post-infection period).

with monensin (0.1 uM) for 20 hr. The values shown are estimated percentages of the average value of binding indices obtained with untreated, uninfected cells. The results indicate that at 0.1 uM monensin, binding of bacteria to both uninfected and RSV-infected HEp-2 cells was increased at ratios, 80, 160, 320, 640 and 1280 bacteria:cell; however, the increase was not statistically significant. The effect was not significant for individual ratios tested.

Results from samples incubated with monensin at 0.05 and 0.2 uM for 20 hr did not differ from those obtained with monensin at 0.1 uM (Fig 7.4). Longer incubation with monensin (30 hr) at 0.1 uM, however, increased the binding greatly to both un-infected and RSV-infected cells. It did not, however, alter the pattern of increased binding to RSV-infected cells compared with uninfected cells (Fig 7.5).

7.3.2.3. Effect of monensin on detection of viral glycoproteins on surface of infected cells: assessed by flow cytometry

Data from immunofluorescence assays with flow cytometry for detection of F and G glycoproteins on the surface of infected cells show that incubation with monensin at 0.1 uM for 20 hr reduced the detection of G glycoprotein significantly ($P<0.05$), whereas this treatment did not affect F glycoprotein. The effect on G glycoprotein was proportional to the dose of monensin used. The longer incubation (30 hr) with monensin at 0.1 uM resulted in decreased detection of both the antigens, but G glycoprotein was more affected (Fig 7.6).

7.3.3. Effect of periodate on bacterial binding and detection of viral glycoproteins

7.3.3.1. Effect on bacterial binding

Analysis of data from 7 experiments with uninfected and RSV-infected HEp-2 cells

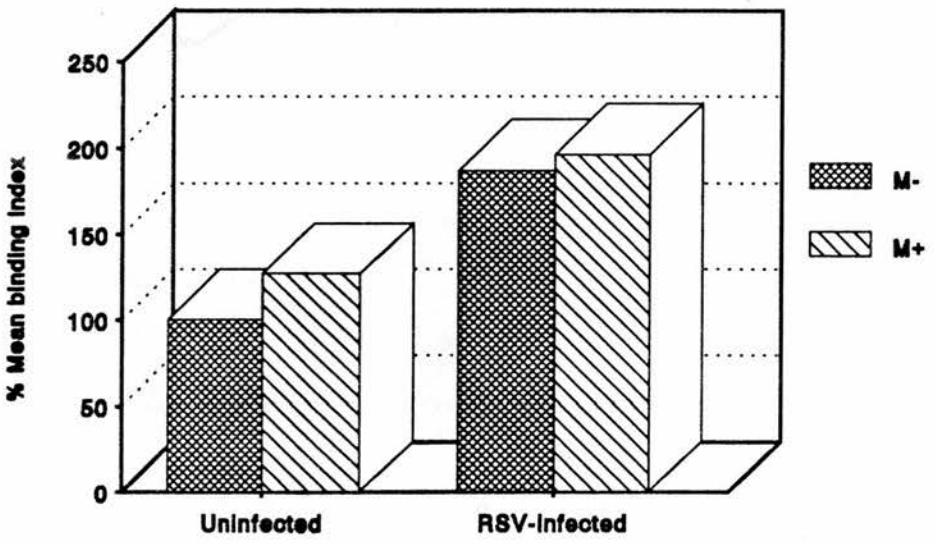

Figure 7.3. Effect of monensin (0.1 uM) on binding of meningococci to uninfected and RSV-infected HEp-2 cells in 7 experiments. (M = monensin treatment)

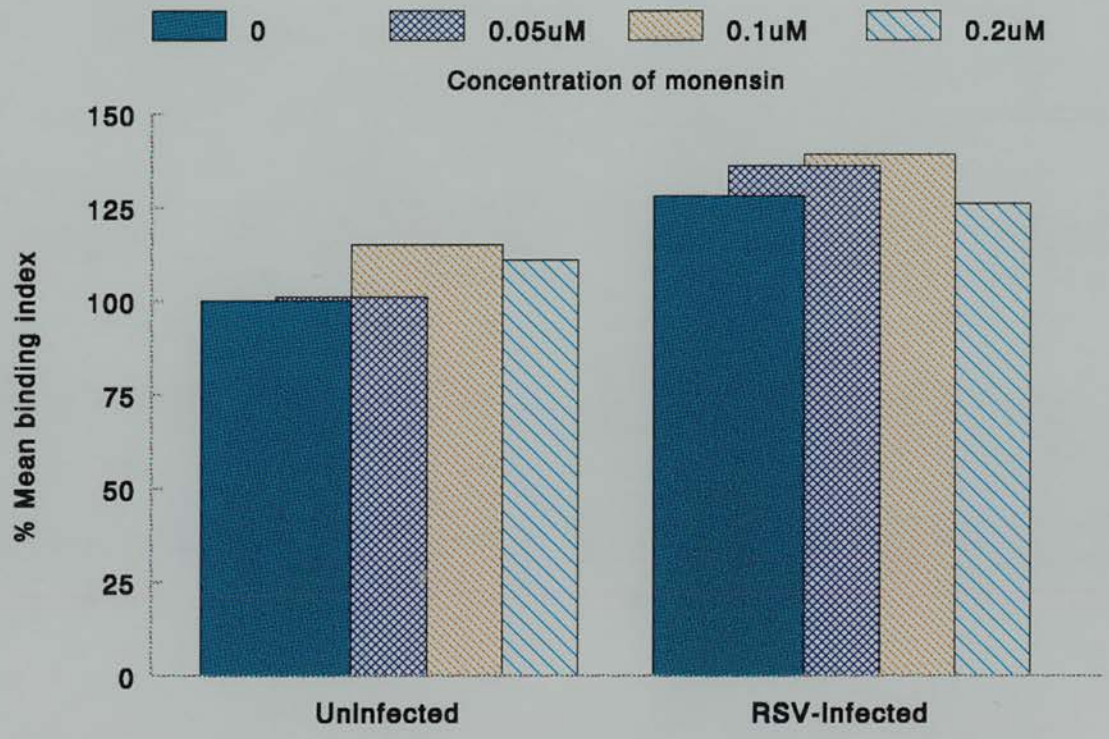

Figure 7.4. Binding of meningococci to uninfected and RSV-infected HEp-2 cells incubated with different concenrations of monensin.

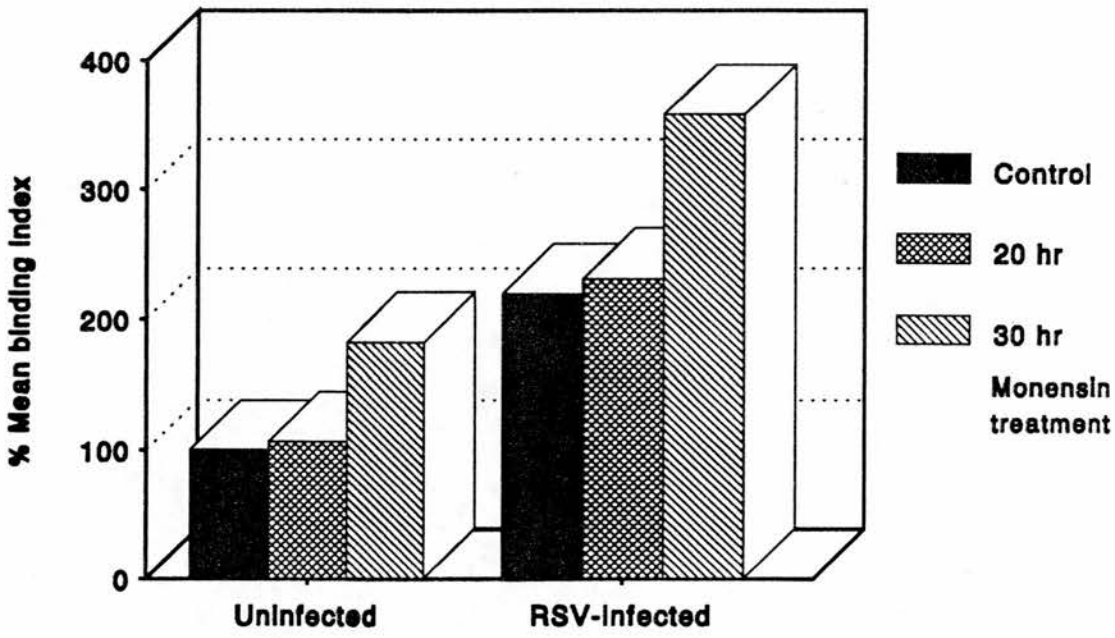

Figure 7.5. Binding of meningococci to uninfected and RSV-infected HEp-2 cells incubated with monensin (0.1 uM) for different periods.

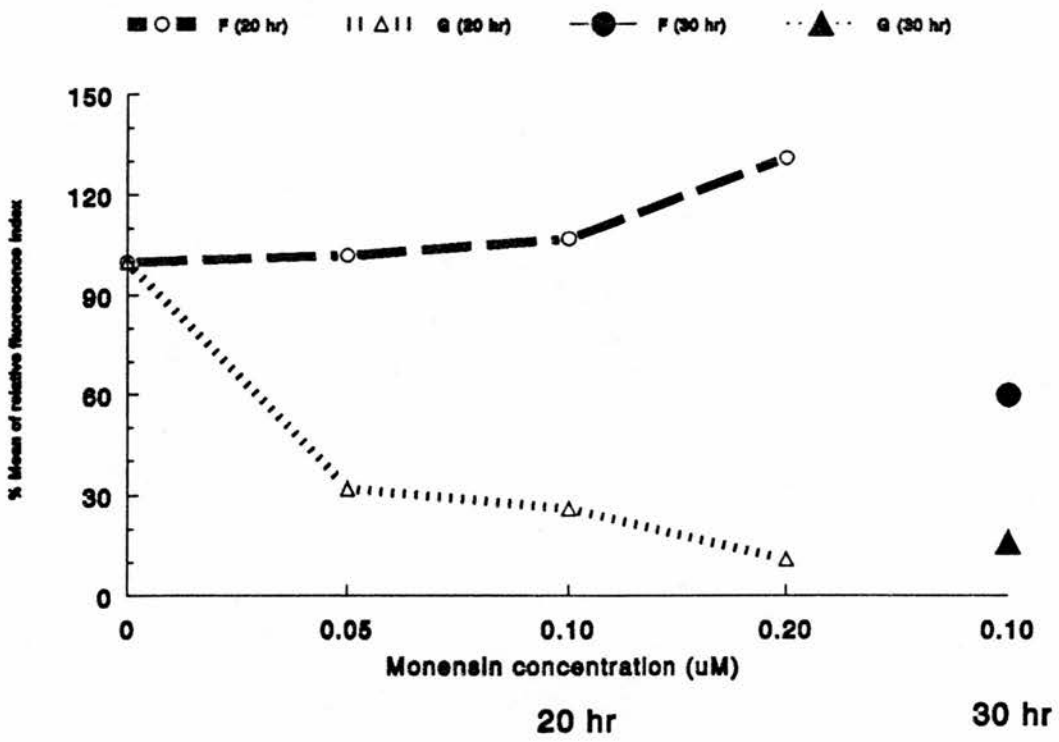

Figure 7.6. Detection of F and G glycoproteins following treatment of RSV-infected HEp-2 cells with various dilutions of monensin for different incubation periods.

treated with periodate for 5 min indicates that at 1.0 uM, periodate did not affect the bacterial binding significantly at ratios of 100, 200 and 400 bacteria:cell. Both uninfected and RSV-infected cells treated with periodate at 7.5 uM bound more bacteria compared with untreated cells at all ratios of bacteria:cell ($P<0.001$). Periodate treatment did not alter the statistically significant increase in bacterial binding to RSV-infected cells compared with uninfected cells (Fig 7.7). Longer incubation (20 min) of the cells with periodate at 1.0 uM resulted in a similar pattern of increased bacterial binding to both uninfected and RSV-infected cells (Fig 7.8).

7.3.3.2. Effect of periodate on detection of G glycoprotein on RSV-infected cells

At 1.0 uM periodate resulted in a small decrease in the detection of G glycoprotein. The effect was greater with periodate at 7.5 uM (Fig 7.9).

7.3.4. The effect of neuraminidase on bacterial binding and detection of viral glycoproteins

Data from two experiments with HEp-2 cells incubated with neuramindase indicate that the enzyme increased bacterial binding to both uninfected and RSV-infected HEp-2 cells at ratios of 100, 200 and 400 bacteria:cell (Fig 7.10). There was a slight reduction in detection of G glycoprotein on RSV-infected cells that were treated by the enzyme (Fig 7.9).

7.4. Discussion

Because carbohydrate antigens on epithelial surfaces often act as receptor for bacterial adhesins, the present studies were carried out to determine if carbohydrate moieties of the G glycoprotein of RSV were involved in binding of meningococci.

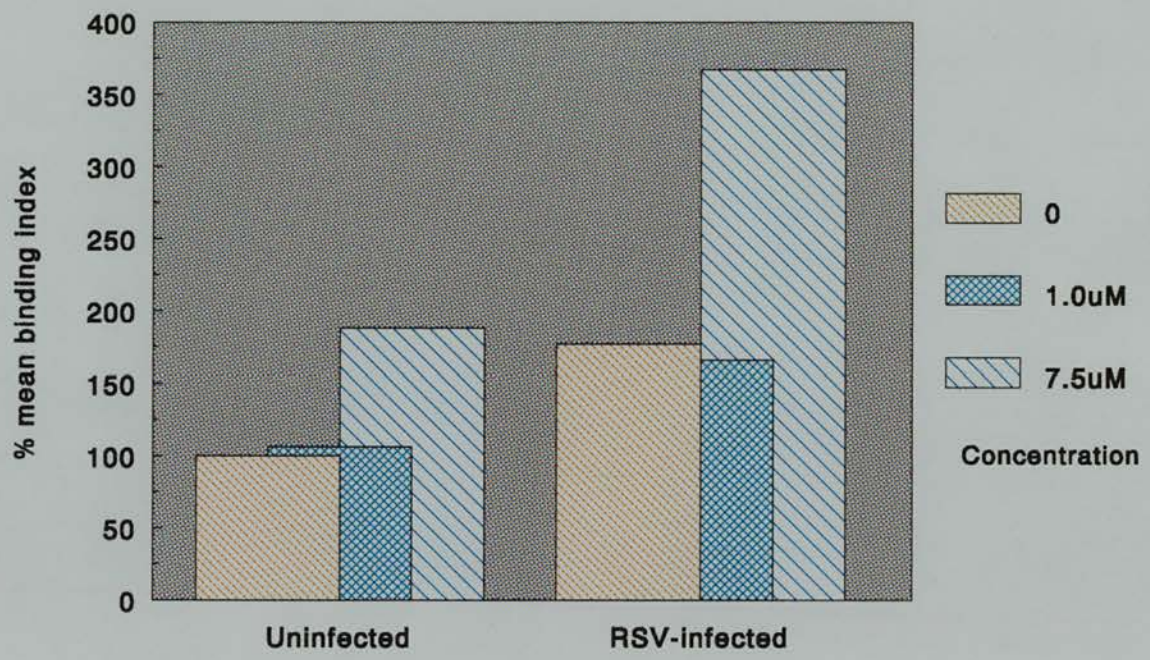

Figure 7.7. Binding of meningococci following treatment of uninfected and RSV-infected HEp-2 cells with periodate at different concentrations.

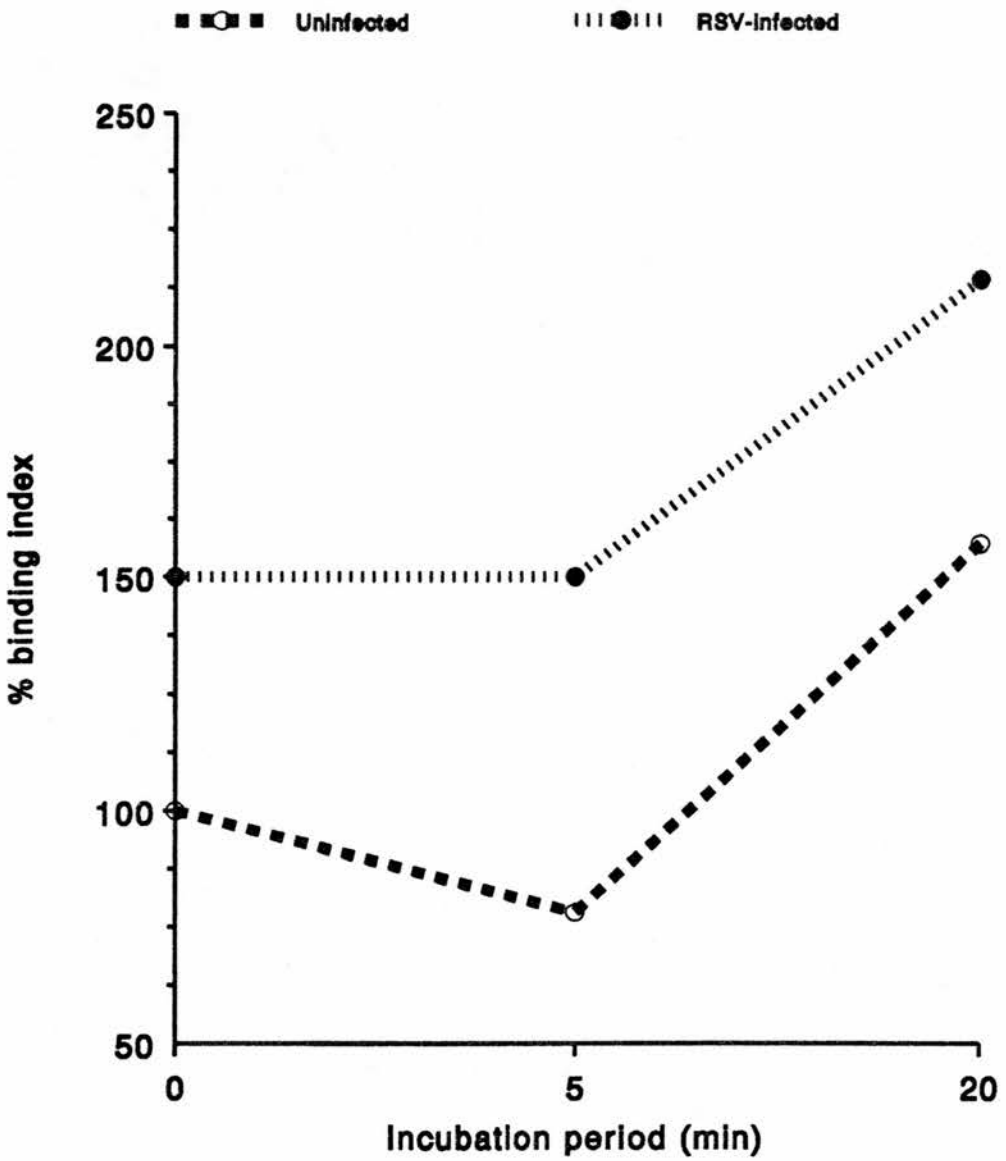

Figure 7.8. Binding of meningococci following periodate (1 uM) treatment of uninfected and RSV-infected HEp-2 cells for different incubation periods.

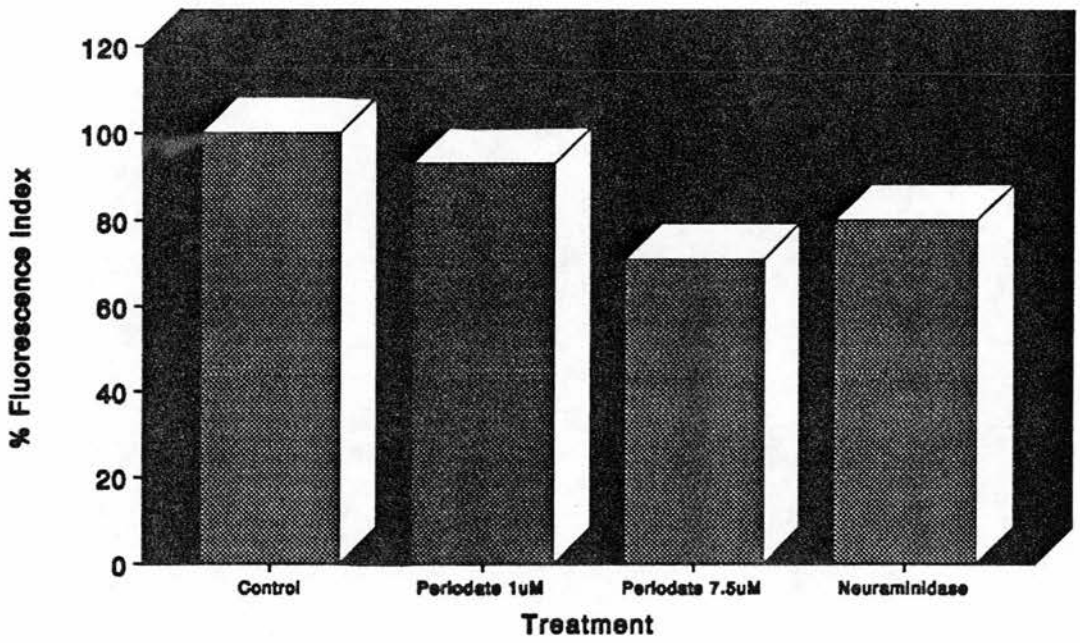

Figure 7.9. Detection of G glycoprotein on RSV-infected cells following treatment with periodate (2 uM or 7.5 uM) or neuraminidase.

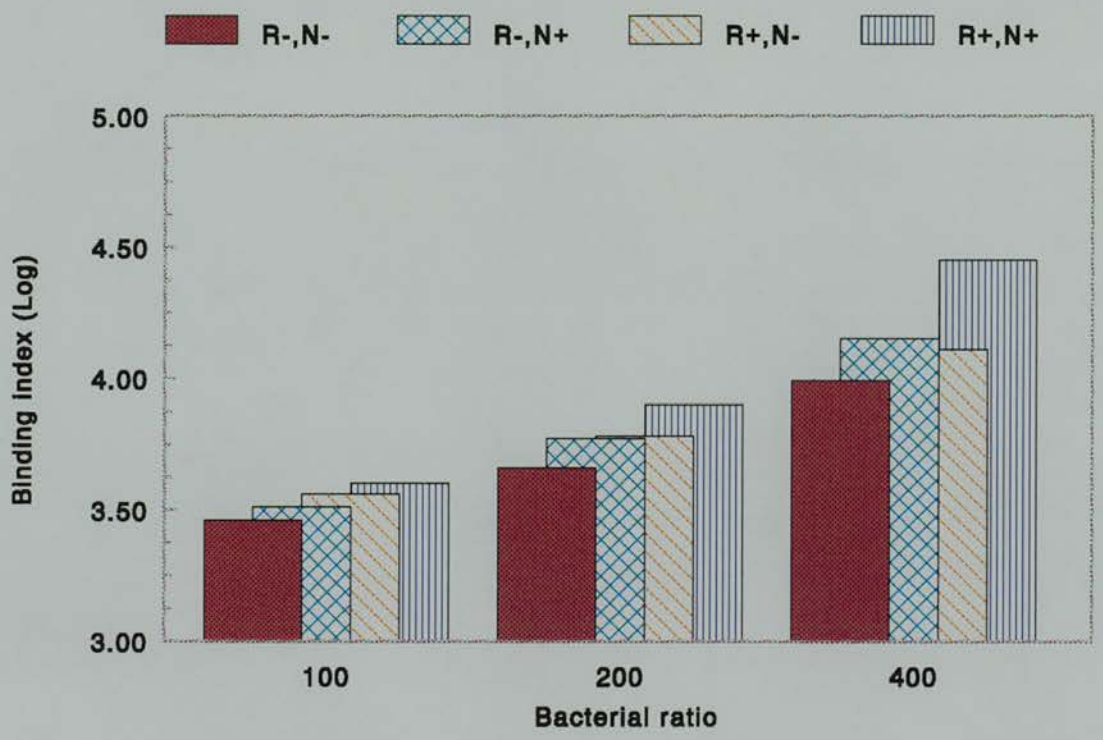

Figure 7.10. Binding of meningococci following treatment of uninfected and RSV-infected HEp-2 cells with neuraminidase in 2 experiments. (R = RSV-infection, N = neuraminidase treatment)

Loss of O-linked sugars of glycoproteins caused by monensin resulted in a decrease in detection of anti-G-defined epitopes. This could be due to monoclonal anti-G that might recognize sugars moieties on G glycoprotein or decreased mean levels of expression of G glycoprotein in the absence of O-glycosylation compared with that observed under conditions of full sugar addition [Wertz, 1989]. The detection of anti-F-defined epitope was not affected. F glycoprotein is involved in formation of syncytia in RSV-infected cell monolayers. The lack of effect of monensin on F glycoprotein was also shown by an un-altered formation of syncytia in monolayers that were treated with monensin during the post-infection period.

Removal of sialic acid and some internally placed neutral sugars from the molecules and oxidation of small numbers of aminoacids by treatment of cells with high concentration (7.5%) of periodate reduced the binding of the anti-G monoclonal antibody. Removal of sialic acid with neuraminidase had similar effects. Conversely, removal of the terminal two sugars from sialic acid following mild treatment of cells with periodate did not affect the detection of G glycoprotein. Loss of O-linked sugars or sialic acid of the G glycoprotein with resultant decrease in detection of anti-G-defined epitope suggest that the monoclonal anti-G was partially directed to sugars in the molecule.

These reagents caused changes in bacterial binding to both un-infected and RSV-infected HEp-2 cells. Whereas loss of O-glycosylation due to monensin treatment of uninfected and RSV-infected HEp-2 cells for 20 hr increased the binding but not significantly, the binding was significantly increased following longer treatment periods (30 hr). Biochemical effects of longer treatment are not documented, but could result from disruption of molecules causing gross changes in the cell morphology (Fig 7.1). Removal of sialic acids and possibly some internal sugars from the molecules and oxidation of some amino acids due to periodate (severe treatment) also increased binding to uninfected and RSV-infected cells.

The results suggest that the carbohydrate moieties affected by treatment with monensin, periodate or neuraminidase are not involved in enhanced binding of meningococci observed with RSV-infected cells. These findings suggest that protein moieties are involved in bacterial binding. Sugars attached to these epitopes might cause steric hindrance to binding. Since the enhancing effect of removal of sugars on the binding was observed with both uninfected and RSV-infected cells, it is not clear whether the effect was attributed to changes in receptor(s) native to cells alone. The proportional increase in binding to uninfected cells due to treatment was not greatly different from that in infected cells. It is, however, likely that protein moieties of the G glycoprotein were involved in binding since exclusion of sugars from the G glycoprotein did not cancel the enhancing effect of viral infection on bacterial binding.

The yield of infectious RSV particles from monensin treated cells is severely reduced [Satake *et al.*, 1985] suggesting O-glycosylation is important in production of infectious particles. The absence of O-linked sugars might affect the configuration and maturation of G glycoprotein [Wertz *et al.*, 1989]. The role of sugars of the G glycoprotein in the attachment of RSV to epithelial cells was, however, not questioned in these studies. In the present study, monensin-treated HEp-2 cells, devoid of O-linked sugars, were not permissive to RSV suggesting that virus-cell association was dependent on O-linked carbohydrates present on the surface of HEp-2 cells. In that case, carbohydrate moieties of G glycoprotein might not directly take part in its attachment function.

In conclusion, these results suggest that protein moieties of G glycoprotein of RSV expressed on infected HEp-2 cells might be involved in attachment to meningococci.

General Discussion

Results of the experiments have been discussed in detail in previous chapters. This chapter summarizes the conclusions with reference to the validity of experimental methods and suggests further studies.

8.1. Secretor status, bacterial meningitis and viral infections

Non-secretion of ABO blood group antigens has been reported to be one of the host factors associated with bacterial meningitis. One objective of the study was to test the hypothesis that non-secretors might be more susceptible to respiratory viral infection. The hypothesis was based on epidemiological evidence that viral infections might be predisposing factors for meningitis or carriage of bacteria associated with meningitis. In contrast to the prediction, secretors were over-represented among the hospitalized patients with disease due to respiratory viruses. The role of Le^b and/or H-type 1 antigens present on cells of secretors as receptors for RSV was examined. Molecules with Le^b or H type 1 determinants did not inhibit RSV-infection of HEp-2 cells.

8.2. Binding of bacteria to virus-infected cells

Bacterial binding to mucosal cells is an initial step in the pathogenesis of invasive disease. The second objective was to assess the effect of RSV-infection of HEp-2 cells on binding of bacteria responsible for meningitis. RSV-infected HEp-2 cells bound significantly more bacteria than uninfected cells. The increased binding was

not associated with a change in the expression of Lewis or H blood group antigens on RSV-infected cells.

8.2.1. A criticism of binding studies

Bacterial association with host mucosal surfaces is affected by a large number of factors. Components of the lining mucus gel, substrates for bacterial growth or dietary lectins can competitively bind to bacterial adhesins and modify binding to specific receptors on cells. The presence of endogenous microflora on the mucosa, local antibodies or adherence of invading pathogens to secreted molecules (e.g., blood group antigens or fibronectin) can also affect bacterial binding to mucosal cells. Although association with the mucosa is often important for bacteria for colonization or disease, this could also result in effective elimination by the host by shedding of mucosal cells. A single *in vitro* binding assay analysing an adhesin-receptor interaction dissociated from the other relevant *in vivo* conditions is of limited value for determining correlations between bacterial adhesion and pathogenicity. Alternatively the interdependence of individual host and virulence factors can be reproduced in animal models. Animal models could, however, be equally criticised if used to examine human pathogens such as bacteria causing meninigits.

Another objection to *in vitro* models for binding studies is that bacteria often possess more than one surface component for binding to cells. Binding assays would, nevertheless, be valid for determination of some of the various adhesive mechanisms that a given bacterium can exhibit. A correlation between *in vitro* adherence and *in vivo* infectivity has been shown for a variety of bacterial pathogens [reviewed by Beachey, 1981]. The model used in the present studies is relevant to *in vivo* infections; the viral glycoproteins expressed on RSV-infected HEp-2 cells are also expressed on epithelial cells of virus-infected mucosa.

The effects of subculturing on bacterial phenotype and FITC-labelling of bacteria on binding to cells has been discussed in chapter 5.

8.2.2. Role of viral glycoproteins in bacterial binding

The experimental approach for determination of cell receptors for bacteria should ideally involve inhibition of binding by specific antibodies or by receptor analogues. Use of mutant cells and bacterial strains deficient in the proposed interacting ligands are a complementary approach. Enzymatic or chemical modification of cells or bacteria help to determine the chemical group of the molecule important in binding.

Results from the assays with antibodies to viral surface glycoproteins suggested that F glycoprotein was not associated with enhanced bacterial binding and that G glycoprotein might be a candidate. Viral glycoproteins could not be used in binding inhibition studies since the glycoproteins in purified form were not available. The use of G and F glycoproteins in these assays might also be complicated due to interaction of these glycoproteins with HEp-2 cells. The surface of the RSV particles is characterized by the presence of the two glycoproteins. The successful attachment of the virus particles to bacteria (Chapter 6) suggested viral glycoproteins might be receptors for bacteria.

Lectins with sugar binding activity have been demonstrated in human epithelial tissues [Sparrow *et al.*, 1987]. Although carbohydrates usually act as cell surface receptors for bacteria, the evidence presented in chapter 7 from experiments with enzyme- or chemically treated cells suggested that protein moieties are involved in binding of meningococci to HEp-2 cells or RSV-infected HEp-2 cells.

Monoclonal anti-G and meningococci competed for the receptor on RSV-infected HEp-2 cells but did not completely inhibit each other (Chapter 6). Anti-G attached to an epitope which appeared to consist of both sugar and protein since loss of sugars

from G glycoprotein resulted in reduced detection by anti-G but did not abolish it (Chapter 7).

Meningococci possess N-acetyllactosamine structures in their lipooligosaccharide (LOS) antigens which have been proposed as adhesins for binding to cellular lectins [Mandrell *et al.*, 1988]. The evidence that meningococci treated with mannose agglutinated yeasts [Mirelman *et al.*. 1980] supports this finding.

Hydrophobicity is one of the important attractive forces in bacterial binding. G glycoprotein of RSV is not overtly hydrophobic. A region of maximum hydrophobicity lies between 41 and 63 residues from the N-terminus and is considered the best candidate for membrane anchoring [Wertz *et al.*, 1985]. Another smaller, conserved, hydrophobic segment exists from residues 164 to 176. This epitope might be the attachment site of the virus [Johnson *et al.*, 1987]. It is devoid of any site for N- or O-glycosylation [Wertz *et al.*, 1985]. The virus is unable to infect HEp-2 cells that have lost O-linked sugars due to treatment with monensin (Chapter 7). This suggests that viral structures interacting with carbohydrate receptor epitope(s) are protein in nature.

The hydrophobic nature of the attachment domain between 164 to 176 might reduce its accessibility to antibodies. Monoclonal antibodies to G glycoprotein partially neutralized the infectivity of RSV [Anderson *et al.*, 1989]. The incomplete inhibition of bacterial binding by monoclonal anti-G (Chapter 6) might be due to relative inability of antibodies to interact with the hydrophobic epitope on the G glycoprotein.

There are many potential sites for O-glycosylation on the G glycoprotein. These sites might be inaccessible to enzymes. A number of bacteria recognize lactosylceramide (a bilayer-close lipid-bound epitope) which is not normally accessible for galactose oxidase [Bock *et al.*, 1988]. It is proposed that bacteria might prove better than single molecules of enzymes to reach an internalized epitope by inducing lateral diffusion of

surface components that are masking the epitope. Bacterial enzymes such as protease of pathogenic meningococci [O'Reilly and Bhatti, 1986] might also help bacteria to reach the internalized epitopes by stripping off surface structures.

8.3. Implications of the findings

It is evident from the difference in the incidence of bacterial meningitis and the carriage rate of the bacteria responsible for meningitis that nasopharyngeal colonization is not sufficient for the disease. There must be qualitative or quantitative changes in the nature of the host-parasite interactions that lead to invasive disease. Binding of pathogens in overwhelming numbers might override the host clearance mechanisms. Invasion might result from establishment of a closer relationship through a different binding mechanism. The enhanced binding of bacteria to RSV-infected cells through ligands other than pili indicate that infection with RSV might be one of the factors contributing to increased colonization of bacteria leading to invasive bacterial disease.

Identification of components on virus-infected cells involved in bacterial binding might be helpful in the search for bacterial epitopes that are involved in colonization and that might induce immunity. Isolation and characterization of the human immune response to the adhesins or the bacteria that bind to glycoprotein might provide a new approach to vaccination against meningococci.

Binding of RSV particles and G glycoprotein in secretions to bacteria in individuals with RSV-infection might provide bacteria with a new adhesin to bind to mucosal cells. Circulatory complement fixing antibodies to the virus might, in contrast, contribute to bacterial killing mechanisms. RSV-infection might predispose to bacterial meningitis in young children during primary virus infection only. The search for an appropriate vaccine against RSV-infection could therefore be relevant to

the prevention of bacterial meningitis.

8.4. Further studies

Flow cytometry is a reproducible, rapid and objective method and can be conveniently used for analysis of binding of FITC-labelled bacteria in future studies. The method, however, could be modified by using labels of two different colours. Analysis of binding of FITC-labelled (green) bacteria to cells in cultures with a mixed population of uninfected and virus-infected cells could be simplified by using a red fluorescent dye conjugated to antibodies to viral structures. A hypothetical histogram from this analysis is illustrated in Figure 8.1. With this technique many variations in the experimental methods (Chapter 5) such as errors in counting cells in different samples and phasic variations in the samples can be avoided.

The other difficulty in these binding studies with flow cytometry is the inability to express the mean fluorescence obtained from FITC-labelled bacteria bound to cells as an actual number of bacteria per cell since the fluorescence emitted by one bacterium is not known. The flow cytometer is equipped with a sorter system in which cells from a specified window drawn on a histogram can be isolated. Cells could be sorted for each level of mean fluorescence and examined by light microscopy for average number of bacteria per cell. This relationship might be applied to determination of the numbers of bacteria bound per cell.

There are antigenic and structural variations in the G glycoprotein of different strains of RSV while only few variations are reported for the F and other viral proteins [Walsh and Hruska, 1983; Ward *et al.*, 1984; Johnson *et al.*, 1987; Walsh *et al.*, 1987]. G glycoprotein of 18573 strain (a subgroup B strain) has a lower molecular weight of 78K compared with 84K of the Long strain (a subgroup A strain, as is the Edinburgh strain used in the present studies) [Walsh *et al.*, 1987]. The amino acid

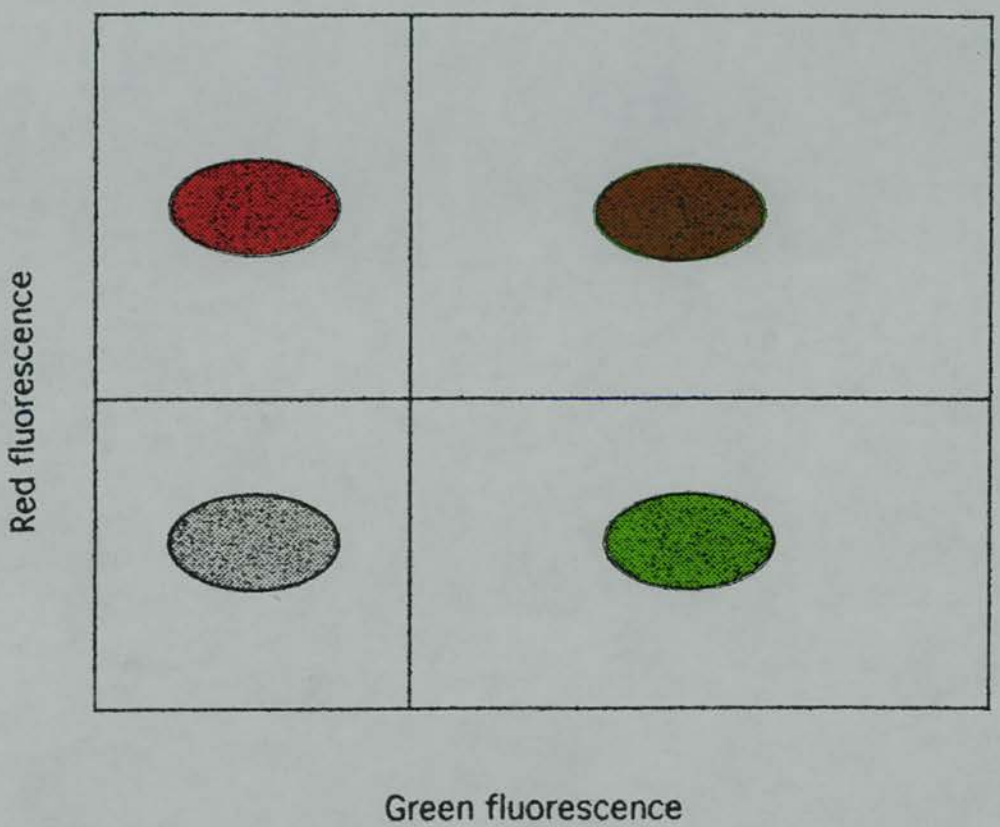

Figure 8.1. A hypothetical histogram from a flow cytometric analysis of a mixed population of uninfected and virus-infected cells incubated with red fluorescein-labelled anti-virus antibodies and green fluorescein-labelled bacteria.

homology between these two strains is 53% [Johnson *et al.*, 1987]. Despite these differences, the secondary and tertiary structure of G glycoprotein of different groups are similar. Cysteine residues (n=4) in the polypeptide occupy conserved sites in the region of residues 173 to 186 that determine the shape of the glycoprotein. The hydrophobic region proposed to be the epitope that attaches to cells is also conserved in different RSV strains [Satake *et al.*, 1985; Johnson *et al.*, 1987]. Evidence that this conserved hydrophobic epitope is the attachment site for bacteria could be obtained from similar studies with HEp-2 cells infected with other strains of RSV.

Cell lines deficient in the expression of enzymes responsible for glycosylation and mutants transfected with human gene(s) responsible for the enzymes might be an appropriate model to investigate the biochemical structures involved in bacterial adhesion.

HEp-2 cells can be employed to demonstrate the effect of RSV-infection on binding of other bacteria, e.g., *Strep. pneumoniae* responsible for meningitis. Cell culture models can be used to study the effect of infection with other respiratory tract viruses, e.g., rhinovirus, adenovirus and parainfluenza virus, on binding of bacteria.

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10. Appendix

10.1. Flow cytometry

10.1.1. Bitmaps

All the particles (cells or bacteria) detected in the flow cytometer were recorded in a histogram with two parameters, log forward angle (X-axis) and log 90° light scatter (Y-axis). Further analysis for fluorescence was carried out on the main populations of particles selected by bitmaps which excluded debris and clumps. Both the HEp-2 cells and bacteria were detected in one histogram to show the relative positions of the two populations (Fig 10.1). The cells were recorded outside the bitmap used to surround bacteria 10.2.a. The subpopulation of events within this bitmap consisted of 85% (n=10,308) of total particles detected in the sample containing bacteria. Since cell population was not completely visualized on the histogram, the forward angle light scatter-filter was used to reduce the signals for size. The bitmap used for cells is illustrated in Fig 10.2.b. The fraction of cell population recorded within the boundries of the bitmap consisted of 89% (n=2538) of total particles detected. The position of the bitmap (for cells or bacteria) for all the samples in any one experiment was constant.

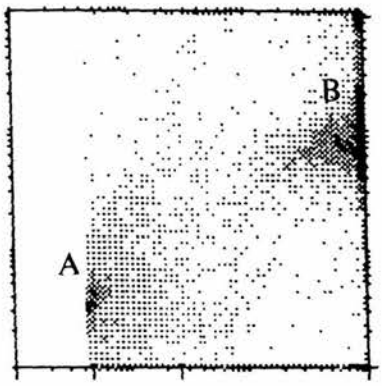

Fig 10.1. Positions of the records obtained from bacteria (A) and cells (B).

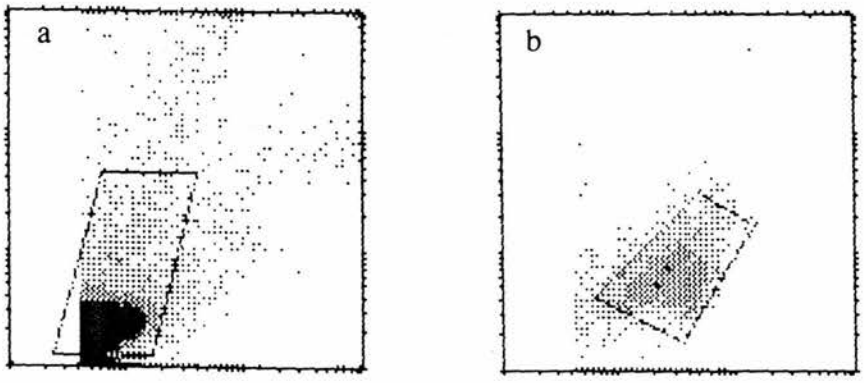

Fig 10.2. Position of the bitmap drawn around the main population of bacteria (a) and HEp-2 cells (b).

10.1.2. Control and test histograms

Drawings in Fig 2 represent typical histograms obtained with HEp-2 cells, uninfected cells or RSV-infected cells incubated with FITC-labelled bacteria.

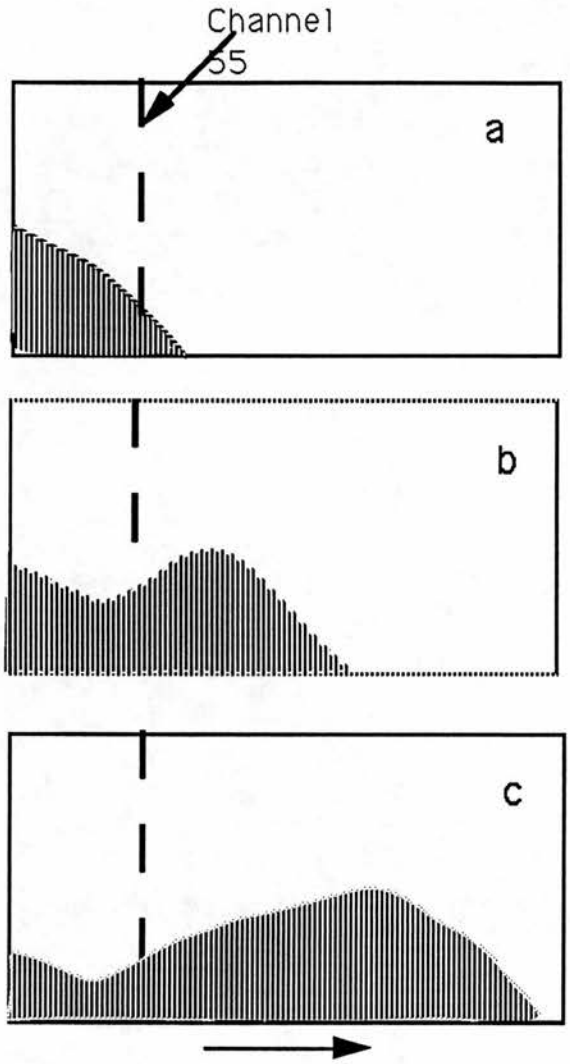

Fig 10.3. Typical histograms showing fluorescence recorded on (a) HEp-2 cells, (b) uninfected HEp-2 cells incubated with FITC-labelled bacteria (250 bacteria : cell) and (c) RSV-infected HEp-2 cells incubated with the bacteria at the same ratio.

10.1.3. Gain

The signals of light scatter and fluorescence from particles are gathered by appropriately placed sensors. Four channels, forward angle light scatter, 90 degree

light scatter and two fluorescence channels, are set up to carry the signals. Each channel is split into two paths, one with a logarithmic scale and the other with a linear scale. The linear scale can be amplified arithmetically by factors (gain) 1, 2, 5, 10, 20, 50 and 100 (Fig 2b).

The amplifications are used to keep the record of events within the limits of the mean channel-histogram with an appropriate spread. For instance, the binding of FITC-labelled bacteria to uninfected cells at a given ratio might be recorded on the linear scale at gain 5. If bacteria at the same ratio bind to RSV-infected cells in higher numbers, the record of fluorescence of this sample at gain 5 might be out of upper limits of the histogram. Use of a lower gain, 1 or 2, is more appropriate to contain the record within the histogram. [Reference Manual (a)].

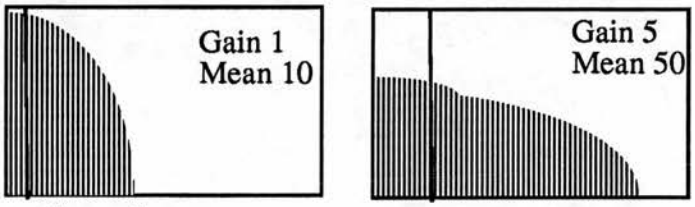

Fig 10.4. Typical example of the mean values of fluorescence of a given fluorescent subpopulation of cells obtained at different gains.

10.1.4. Immunoanalysis programme

Data obtained from the samples can be analysed for the proportion of positive and negative subpopulations by this programme. In the example given in Fig 3 the use of the cursor to determine the percentage of positive cells in the test population results in an underestimation. It is apparent that all the cells in the test sample have moved to the right on the X-axis with relation to the control sample. The immunoanalysis programme uses the information obtained from the negative control histogram to draw a hypothetical histogram representing positive subpopulation from the test sample.

The conditions required for this analysis were satisfied by the data presented in this study: 1) a match region between control and test histograms was established; 2) the histograms with identical channel numbers were subjected to the analysis. [Reference manual (b)].

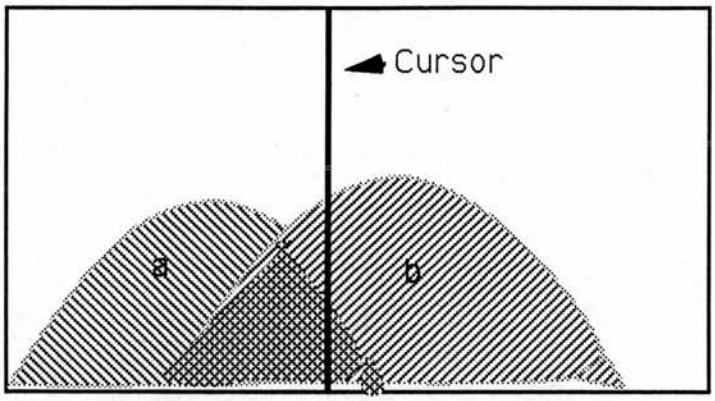

Fig 10.5. Histograms with log green fluorescence from (a) control sample and (b) test sample merged together

10.2. Calculation of binding index

A binding index for each sample in the study was calculated by multiplying the percentage of positive cells and the mean fluorescence of positive subpopulation. In similar studies in which binding of yeasts to epithelial cells was examined., a greater variation was found in percentage values compared with mean values in different experiments [Aly, 1992]. It has been suggested that mean numbers of bacteria attached to whole population of cells provide a more accurate estimate of adhesiveness [Freter and Jones, 1983]. In the present study the data were presented as binding indices (BIs) since the virus infection might affect the percentage of cells binding bacteria and the mean numbers of bacteria bound. Table 1. illustrates calculation of the BIs for one experiment (see 5.3.3.3).

Media used	Uninfected cells			RSV-infected cells		
	%	Mean	BI	%	Mean	BI
NYC	81	21	1701	79	23	1817
GC	80	23	1840	95	21	1995
BBA	92	73	6716	99	84	8316

Table 10.1. An example of calculation of binding indices from an experiment with FITC-labelled C:2b:P1.2 grown on different culture media bound to uninfected or RSV-infected HEp-2 cells at a ratio of 640 bacteria per cell.

10.3. Daily variations in the experiments

There were wide variations in the figures obtained from the BIs in different binding experiments. The factors considered to contribute to these variations and their possible effects on the results are discussed in section 5.4.2. In Table 2 BIs from 6 individual experiments with FITC-lablled bacteria,HEp-2 cells and cells infected with RSV at M.O.I. 1.0 are given. Binding was higher in some experiments compared with others, but the effect of RSV-infection of cells in enhancing binding of bacteria was, however, consistent.

Binding indices

Bacteria/ cell	80		160		320	
Experi-ments	Uninfected	RSV-infected	Uninfected	RSV-infected	Uninfected	RSV-infected
1	180	241	274	291	586	737
2	162	100	167	292	219	618
3	181	163	170	308	-	-
4	51	76	258	1060	845	1655
5	486	613	1617	2371	2260	4396
6	61	86	97	130	168	368

Table 10.2. Binding indices for six experiments with FITC-labelled C:2b:P1.2 and HEp-2 cells or HEp-2 cells infected with RSV at M.O.I. 1.0 (24 hr post-infection).

10.4. **References**

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radiographs are commonly inadequate for diagnosing conditions such as spondylitis, metastasis at the base of a pedicle, and listhesis,[18] and therefore, apparently normal routine radiographs should not be considered adequate if the clinical picture indicates important disease.

It is surprising that so few patients were fully examined and that so many were not examined before the radiographs were requested. Although patients may forget much of what their general practitioner tells them in a consultation, we consider that most will remember whether or not they have been examined. At this unit most patients will have had their examination performed within a few days of the request so that elapsed time does not become a major factor in accuracy of recall. We thus believe that our figures are a reasonable approximation to the truth. It can only be that most of the doctors in our sample currently make their decision to request radiographs based on the patient's history and that the examination findings are unlikely to alter this decision one way or the other. We hope that dissemination of the college guidelines will help to modify this decision making process.

Few of the general practitioners we contacted were aware of the college guidelines, and most expressed an interest in seeing them. Radiologists should strive to ensure that their local general practitioners are informed of the guidelines and should discuss their implications with them.

Despite the pessimism of some authors,[19] there is evidence that educating clinicians about radiology can reduce the number of unnecessary examinations,[20 21] and in view of the many patients referred to our department for lumbar spine radiography, we hope that widespread acceptance of the guidelines will result in optimal use of radiological services. We also believe that there is a need for a guided increase in public awareness regarding the radiation engendered in diagnostic radiology. Though we do not wish to cause unnecessary concern, the community as a whole would benefit from a reduction in medical radiation that might follow reduced demand from patients to have radiographic examinations for painful but benign conditions.

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Association between secretor status and respiratory viral illness

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Abstract

***Objective*—To determine whether non-secretion of blood group antigens is associated with respiratory virus diseases.**

***Design*—Study of secretor status in patients with respiratory virus diseases determined by an enzyme linked immunosorbent assay (ELISA) developed to identify Lewis (Le) blood group antigen phenotypes (Le^a non-secretor; Le^b secretor).**

***Subjects*—Patients aged 1 month to 90 years in hospital with respiratory virus diseases (584 nasal specimens).**

***Main outcome measures*—Criteria for validation of ELISA (congruence between results on ELISA testing of 1155 saliva samples from a previous study and previously established results on haemagglutination inhibition (HAI) testing, proportions of Le^a, Le^b, and Le^- phenotypes in 872 samples of nasal washings from a previous study compared with the normal population). Secretor status of patients determined by ELISA and viruses isolated.**

***Results*—Agreement between HAI and ELISA for 1155 saliva samples was 97%. Lewis antigens were detected by ELISA in 854 (97·9%) of nasal washings (Le^a 233 (26·7%), Le^b 621 (71·2%), and Le^- 18 (2·1%)) in proportions predicted for a northern European population. Secretors were significantly overrepresented among patients from whom influenza viruses A and B (55/64, 86%; $p<0·025$), rhinoviruses (63/72, 88%; $p<0·01$), respiratory syncytial virus (97/109, 89%; $p<0·0005$), and echoviruses (44/44, $p<0·0005$) had been isolated compared with the distribution of secretors in the local population.**

***Conclusion*—Secretion of blood group antigens is associated with respiratory virus diseases.**

Introduction

Susceptibility to a variety of bacterial and superficial fungal infections is associated with the genetically controlled inability of individual subjects to secrete the water soluble form of the ABO blood group antigens into body fluids (non-secretion).[1-7] Non-secretors are also significantly overrepresented among patients with some autoimmune diseases for which infectious triggers have been proposed.[8-12] Although studies of associations between ABO blood groups and susceptibility to natural or experimental viral infections have been reported,[13-15] there are no published studies of secretor status and viral infection. In this study we tested the hypothesis that non-secretors might also be at increased risk of viral illnesses.

Because the quantities of material available from

patients with viral diseases were too small to determine secretor status by the usual haemagglutination inhibition method,[16] we developed an enzyme linked immunosorbent assay (ELISA) to detect Lewis blood group antigens in the specimens. Non-secretors express only Lewis^a (Le^a) antigen and secretors express Lewis^b (Le^b) antigen on their red blood cells and in their body fluids.

Materials and methods

ELISA FOR LEWIS ANTIGENS

To detect the presence of Le^a and Le^b blood group antigens in body fluids by ELISA wells of polystyrene microtitre plates (Dynatech, Billingshurst, Sussex) were coated overnight at 4°C with 100 µl of monoclonal Le^a antibody (LM 112/161) or 100 µl Le^b antibody (LM 129/81 anti-Le^{bL}) (provided by R H Fraser, Glasgow and West Scotland Blood Transfusion Service). Le^a antibody was diluted 1 in 25 and Le^b diluted 1 in 20 in 50 mM sodium carbonate buffer (pH 9·6). All further procedures were carried out at room temperature except when stated otherwise. The wells were washed three times with 0·1 M phosphate buffered saline containing 0·1% (wt/vol) bovine serum albumin and 0·05% (vol/vol) Tween 20 (washing buffer). The wells were blocked with 150 µl of phosphate buffered saline with 1% (wt/vol) bovine serum albumin (blocking buffer) for 15 minutes. The buffer was removed and the wells washed twice with washing buffer.

Dilutions of saliva from known secretors or non-secretors were used in each plate as controls. Test wells contained 100 µl of the specimen (saliva, nasal washings, or respiratory secretions) which had been boiled for 30 minutes to inactivate enzymes, bacteria, or viruses. Samples of saliva were diluted 1 in 20 in blocking buffer for detecting Le^b antigen and 1 in 100 for detecting Le^a antigen. The more dilute nasal washings or respiratory specimens were diluted 1 in 10 in blocking buffer to detect Le^a antigen but were used undiluted to detect Le^b antigen.

After incubation for one hour the wells were washed three times and 100 µl of polyclonal goat anti-Le^a antibody (Behring, Marburg, West Germany) diluted 1 in 500 in blocking buffer or 100 µl of polyclonal goat anti-Le^b antibody (Behring) diluted 1 in 250 in blocking buffer were added to the wells of the appropriate plates. After 30 minutes' incubation the plates were washed three times, and 100 µl of horseradish peroxidase conjugated donkey antigoat immunoglobulin (Scottish Antibody Production Unit, Carluke, Lanarkshire) diluted 1 in 250 in blocking buffer was added. After overnight incubation at 4°C the plates were washed three times and 100 µl of orthophenylene diamine (40 mg in 100 ml 0·1 M phosphate citrate buffer, pH 5) activated with 40 µl hydrogen peroxide (30%) were added to each well. The colour was allowed to develop for 10-15 minutes, and the reaction was stopped by adding 50 µl of 12·5% sulphuric acid to each well.

Absorbance at 490 nm was measured with a plate reader (Dynatech). Samples were tested in duplicate, and the readings were averaged. The average reading for each sample was compared with the results obtained for dilutions of saliva from secretors (diluted 1 in 20) and non-secretors (diluted 1 in 100) in the same plate. Values equal to or above that of the control were considered to be positive. The subjects from whom the samples were obtained were classified as non-secretors if only Le^a antigen was detected or as secretors if Le^b antigen or Le^a and Le^b antigens were detected.

SAMPLES AND CONTROLS

To assess the method we compared the results obtained by the ELISA with those obtained by a haemagglutination inhibition assay[16] for 1155 saliva specimens collected during the Stonehouse survey.[17] Nasal secretions were collected from 26 members of staff whose secretor status and Lewis blood group antigens had been previously determined. The secretions were collected with cotton wool swabs, inoculated into virus transport medium, and the transport medium processed and assayed for presence of Lewis antigens. Nasal washings (n=872) obtained as part of other studies at the Medical Research Council's Common Cold Unit were also tested to determine if Lewis antigens could be detected in diluted nasal secretions. They had originally been obtained from volunteers for assessment of secretory antibodies and had been stored at −20°C for four to five years.

As there is no association between sex and secretor status or sex and the Lewis blood group[18] the distribution of Le^a and Le^b antigens in the local population was determined with red blood cells from 363 women attending antenatal clinics at the Royal Infirmary, Edinburgh, by the standard tube agglutination method with the monoclonal Le^a and Le^b antibodies referred to above in 10% dextran and 2% bovine serum albumin. The results were compared with those of a previous study of the local population in which secretor status of 334 blood donors was determined from saliva by haemagglutination inhibition.[3] Specimens sent to the regional virus laboratory for examination (n=584), mainly from patients aged from 1 month to 90 years in hospital with symptoms of viral disease, were used in this study. These were aspirated respiratory secretions or nasal or throat swabs inoculated into virus transport medium which had been stored at −70°C after culture for virus (table I). The results for the local population and test specimens were compared by the χ^2 test incorporating Yates's correction factor. Odds ratios and 95% confidence intervals were calculated by the exact method.

TABLE I—*Source of specimens for determination of Lewis blood group and secretor status*

Source	Specimen	No
Controls:		
Local antenatal clinic	Blood	363
Local antenatal clinic[3]	Saliva	334
Stonehouse survey[17]	Blood and saliva	1155
MRC Common Cold Unit	Nasal washings	872
Laboratory staff	Saliva, blood, and nasal swabs inoculated into virus transport medium	26
Patients:		
Regional virus laboratory	Nasal secretions OR Swabs inoculated into virus transport medium	584

Results

ASSESSMENT OF ELISA FOR DETECTING LEWIS ANTIGENS IN BODY FLUIDS

Le^a or Le^b antigens or both, were detected by ELISA in 1089 of the 1155 (94·3%) specimens of saliva for which secretor status had been determined by haemagglutination inhibition. The results of ELISA for Lewis antigens and of the haemagglutination inhibition assay agreed for 1058 of the 1089 Lewis positive specimens (97%), 796 Le^b antigen positive (secretors) and 262 Le^a antigen positive (non-secretors). The results for the two assays disagreed for 31 specimens, 27 Le^a antigen positive (but secretors by haemagglutination inhibition) and four Le^b antigen positive (but non-secretors by haemagglutination inhibition). In 66 specimens (5·7%) Lewis antigens were not detected by ELISA: 30 were from secretors and 36 from non-secretors. Lewis phenotype determined by agglutination of red blood cells for 124 of these donors

agreed with the results of the ELISA for 119 (96%) of those tested. The ELISA correctly identified the Lewis antigen present in nasal secretions of all 26 laboratory staff. Among the 872 nasal washings obtained from the Common Cold Unit, Lewis antigens were detected in 854 (97·9%): Le^a antigen in 233 (26·7%); Le^b antigen in 621 (71·2%); and no Lewis antigen in 18 (2·1%). This finding does not differ from the distribution of those phenotypes in most northern European populations.[18]

LEWIS PHENOTYPES OF LOCAL POPULATION

Among 363 blood specimens from the antenatal clinic in which the Lewis antigens were detected, 28% were Le^a antigen positive only and 72% were Le^b antigen positive (tabe II). These results were not significantly different from the proportions of non-secretors (26·6%) and secretors (73·4%) determined in a previous study by haemagglutination inhibition tests of 334 samples of saliva from local blood donors.[3]

LEWIS PHENOTYPE OF PATIENTS WITH VIRAL ILLNESSES

In 192 of the 584 (33%) patients' specimens examined by ELISA not enough Lewis antigen was detected for definite classification: 81 showed borderline readings, and in 111 no antigen was detectable. The results obtained with these specimens most probably reflect degradation of the antigens due to delays in their transportation to the laboratory. No virus was isolated from 38 (20%) of these 192 specimens. Among the 392 specimens in which either or both Lewis antigens were definitely detected, no virus was isolated from 36 (9%) ($\chi^2=12·17$, $p<0·0005$). The proportion of unclassifiable specimens did not vary significantly with respect to isolation of any particular virus. As the specimens giving negative and borderline results for Lewis antigens could not be classified as being from secretors or non-secretors they were eliminated from further analysis.

TABLE II—*Lewis phenotype and secretor status of patients and controls and results of virus culture*

Source of specimen	Virus isolated	Le^{a+b-} (non-secretors) No (%)	Le^{a-b+}/Le^{a+b+} (secretors) No (%)	χ^2	p Value	Odds ratio* (95% confidence interval)
Antenatal clinic controls	103 (28)	260 (72)				
Regional virus laboratory:						
	Influenza	9 (14)	55 (86)	5·74	<0·025	2·42 (1·13 to 5·77)
	A	7 (14)	44 (86)	4·92	<0·05	2·49 (1·06 to 6·76)
	B	2 (15)	11 (85)	0·51	>0·05	2·18 (0·46 to 20·53)
	Parainfluenza	17 (25)	50 (75)	0·13	>0·05	1·17 (0·63 to 2·26)
	Respiratory syncytial virus	12 (11)	97 (89)	12·77	<0·0005	3·20 (1·66 to 6·67)
	Rhinovirus	9 (12)	63 (88)	7·11	<0·01	2·77 (1·31 to 6·57)
	Echovirus	0	44 (100)	15·25	<0·0005	∝ (4·41 to ∝)
	None	9 (25)	27 (75)	0·06	>0·05	1·19 (0·52 to 2·97)

*Odds ratio compared with local controls.

Table II compares the isolation of viruses from non-secretors and secretors. Compared with the local population, there was a significantly higher proportion of secretors among subjects from whom the following viruses were isolated: influenza A virus ($p<0·05$), rhinovirus ($p<0·01$), respiratory syncytial virus ($p<0·0005$), and echoviruses ($p<0·0005$). Although 11 of 13 specimens containing influenza B virus were from secretors, the numbers were too small to be significant. This pattern was not observed for the 67 specimens from which parainfluenza virus was isolated or the 36 from which no virus was isolated. In these two groups of specimens the proportions that were Le^b antigen positive (secretors) and Le^a antigen positive (non-secretors) were similar to those of the local population.

Discussion

Determination of Lewis phenotype is a good control for haemagglutination inhibition assays for ABO antigens which have been the standard method for determining secretor status. Agreement between the Lewis phenotypes and results of the haemagglutination inhibition assay for 1089 saliva specimens was 97%. "False secretors," of Le^a phenotype but secretors by haemagglutination inhibition, were the predominant mismatched pairs (27/31, 87%). Results of a previous study indicate that these are most likely the result of contamination of saliva by blood owing to poor oral hygiene or periodontal disease among these subjects.[17] Dilution of small samples to provide enough material for the haemagglutination inhibition test is probably the source of the small proportion (0·004%) of "false non-secretors," who are of Le^b phenotype but non-secretors by haemagglutination inhibition. The ELISA method eliminates the problem of contamination of non-secretor saliva by red blood cells and it can be carried out on smaller volumes than those needed for haemagglutination inhibition. The method also detected Lewis antigens in 854 (98%) of the 872 specimens from the Common Cold Unit, indicating that the method can be used to detect these antigens in diluted nasal secretions.

The nasal washings from volunteers at the Common Cold Unit were originally collected for determining secretory antibody titres and were frozen soon after collection, which would preserve the Lewis antigens; analysis of the results found the expected proportion (2%) of specimens negative for Lewis antigen. The high proportion of specimens from patients in hospital with viral illness for which borderline readings were obtained or in which no Lewis antigen was detected might be due, in part, to collection techniques and time taken for transportation and processing the specimens. Blood group antigens cannot be reliably detected in saliva kept overnight at room temperature. There was a significantly higher proportion of unclassifiable specimens from which no virus was isolated (20%) compared with the proportion of specimens in which Lewis antigens were definitely detected but from which no virus was isolated (9%) ($p<0·0005$). The proportion of unclassifiable specimens was not greater among the very young age groups (<24 months), from whom nasopharyngeal secretions are usually obtained. Although the Lewis antigens were correctly identified in all the samples of transport medium inoculated with nasal swabs obtained from 26 laboratory staff, secretions provide a larger quantity of material for isolating virus and detecting Lewis antigen.

Previous studies found non-secretion to be associated with various bacterial diseases and superficial yeast infections and with carriage of some pathogenic bacteria or yeasts.[19-21] These findings contrast with our present finding in which secretors were overrepresented among those patients with significant symptoms of respiratory illness and from whom influenza A virus, rhinovirus, or respiratory syncytial virus were isolated. Secretors were also significantly overrepresented among those from whom echoviruses were isolated; these patients, however, had various illnesses including meningitis, fever, and vomiting. An increase in the proportion of secretors was not associated with isolation of parainfluenza virus or with the group of individual subjects from whom no virus was isolated.

This is the first report of associations between secretion of blood group antigens and infectious diseases. There is evidence that the Le^a antigen present in greater amounts on epithelial surfaces of non-secretors might be one of the receptors for *Candida* species,[22] and studies in progress indicate that this might be true for *Neisseria meningitidis*. The hypothesis

that antigens present only in secretors (Le^b) or in higher quantities in secretors (H type 1 in addition to H type 2)[23] might act as one of the receptors for some viruses is under investigation.

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Mode of delivery after one caesarean section: audit of current practice in a health region

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Abstract

***Objective*—To audit the subsequent obstetric management of women who had had one previous baby delivered by caesarean section.**

***Design*—Retrospective analysis of a regional obstetric database.**

***Setting*—Data derived from the 17 obstetric units in North West Thames region.**

***Subjects*—1059 women who delivered a singleton fetus of at least 37 weeks' gestation with a cephalic presentation in 1988 who had a history of one previous caesarean section and no other deliveries.**

***Main outcome measures*—Mode of delivery, postnatal morbidity, and duration of hospital stay.**

***Results*—395 (37%) women were delivered by elective repeat caesarean section and 664 (63%) were allowed a trial of labour. Maternal height and birth weight of the previous infant differed significantly between those who were and those who were not allowed to labour. 471 (71%) of those allowed to labour achieved a vaginal delivery. In individual units there was no significant correlation between the proportion of patients allowed to labour and the rate of the successful trial of labour. There was a trend towards greater success rates in units that allowed a longer duration of labour ($p<0.05$) and units with greater use of oxytocin for augmentation of labour (not significant). Both elective and intrapartum caesarean section was associated with a significantly higher rate of postnatal infection than vaginal delivery (14·7% and 16·0% *v* 3·4%).**

***Conclusions*—In patients with a history of caesarean section there is no evidence that the likelihood of successful vaginal delivery after trial of labour is modified by the proportion of such patients allowed the option of attempted vaginal delivery. Until selection criteria of adequate prognostic value can be identified a more liberal approach to allowing women a trial of labour seems justified.**

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Introduction

The rising incidence of caesarean birth in Britain and elsewhere is a cause for concern both in terms of the associated increase in clinical and social morbidity for the mother and increased cost to the health service. Repeat caesarean section makes a major contribution to the overall rate of caesarean section. One strategy for reducing the rate of caesarean birth, therefore, is to allow women with a history of lower segment caesarean section the option of a trial of labour in their next pregnancy unless there are specific contraindications.

Many studies attest to the safety of a properly conducted trial of labour in women who have previously delivered by caesarean section, and successful vaginal delivery can be expected in around two thirds of such cases. In a comprehensive review Lavin *et al* concluded that a properly managed trial was associated with an acceptably low incidence of scar dehiscence and perinatal mortality.[1] Furthermore, no maternal deaths were identified. This is in contrast to the recognised contribution of repeat elective caesarean section to maternal mortality.[2]

Factors known to influence the outcome of a trial of